YWHAB knockdown inhibits cell proliferation whilst promoting cell cycle arrest and apoptosis in colon cancer cells through PIK3R2

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Abstract. Colon cancer is one of the most common causes of cancer-associated mortality. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β (YWHAB) has been reported to be aberrantly expressed in human colon cancer cells following alltrans retinoic acid treatment. PI3K regulatory subunit 2 (PIK3R2) has also been identified as a gene associated with colon cancer metastasis and tumor progression. The present study aimed to determine the role of YWHAB in colon cancer in addition to its detailed reaction mechanism. The expression levels of YWHAB and PIK3 'A0* R2 before or after transfection of YWHAB interference plasmids or PIK3R2 overexpression plasmids were examined by reverse transcription-quantitative PCR and western blotting. PI flow cytometry, Cell Counting Kit-8 and TUNEL assays were performed to measure the extent of cell cycle progression, proliferation and apoptosis. Additionally, the expression levels of G_1-S cell-cycle transition regulator cyclin D1 and G_1-checkpoint CDK inhibitor p21 and apoptosis marker proteins Bcl2 and Bax were assessed using western blotting. Subsequently, the Monarch Initiative database (https://monarchinitiative.org/) predicted the binding of YWHAB and PIK3R2, following which co-immunoprecipitation assay was utilized to assess their potential interaction. Furthermore, western blotting was performed to examine the expression levels of PI3K/AKT signaling pathway markers. It was revealed that YWHAB expression was upregulated in colon cancer cells compared with HIEC-6 human intestinal epithelial cells. Functionally, YWHAB depletion by transfection of YWHAB interference plasmids was demonstrated to suppress the proliferation of colon cancer cells whilst promoting cell cycle arrest at the G_0/G_1 phase and apoptosis, decreasing cyclin D1 and Bcl2 expression, and increasing p21 and Bax expression. Additionally, YWHAB was verified to bind to PIK3R2 and YWHAB knockdown decreased PIK3R2 expression. PIK3R2 overexpression by transfection of PIK3R2 overexpression plasmids reversed the effects of YWHAB knockdown on cell proliferation, cycle arrest, apoptosis, and apoptotic and cell cycle proteins in colon cancer cells. YWHAB knockdown reduced the levels of p-PI3K/PI3K and p-AKT/AKT and PIK3R2 overexpression also reversed the effects of YWHAB knockdown on the expression of PI3K/AKT signaling markers. In conclusion, these data suggest that YWHAB can activate the PI3K/AKT signaling pathway and participate in the malignant progression of colon cancer by targeting PIK3R2, which provides novel insights into the mechanism of colon cancer.

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Introduction

Colorectal cancer is one of the most prevalent types of cancer and the leading contributor of cancer-associated mortality in both men and women (1). It has been estimated that colorectal cancer resulted in 104,610 new cases and >50,000 deaths in the United States in 2020 (2). In particular, colon cancer is considered to be a single disease entity compared with colorectal cancer and has unique characteristics in terms of treatment, prognostic and metastatic profiles (3,4). According to data released by the American Cancer Society, the incidence rate of colon cancer has reached 10.2% whereas the mortality is ~9.2%, making it one of the most common malignant tumors in the USA (5,6). A previous study has suggested that the risk factors in the etiology and pathogenesis of colon cancer include unhealthy diet, obesity, inadequate physical activity, microbiome and family history (7). Although substantial progress has been made in terms of novel surgical and therapeutic strategies for this cancer, in addition to the development of targeted treatment methods, the overall survival rate of patients with advanced stages of colon cancer remains low and the prognosis remains poor (8,9). Therefore, exploration of independent biomarkers involved in the progression of colon cancer is urgently required.

Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β (YWHAB), also known as 14-3-3β/α, is a member of the highly conserved 14-3-3 protein family (10,11). It has been documented to regulate various cellular processes, including cell proliferation, signal transduction, cell cycle and apoptosis (10,11). Downregulation of YWHAB has been reported to impart suppressive effects on the proliferation of cervical and gastric cancer cells (12,13). Additionally, YWHAB was previously found to be upregulated in colon cancer cells, which is in turn associated with poorer prognosis (14). However, the detailed role of YWHAB in the malignant phenotypes of colon cancer and its potential regulatory mechanism remain poorly understood.

The PI3K regulatory subunit 2 (PIK3R2) gene has been previously identified to be an activator of the PI3K/AKT signaling pathway in the regulation of cell proliferation and apoptosis of various cancer types, including bladder cancer, non-small-cell lung cancer and glioma (15,16). Accumulated evidence has demonstrated that the downstream component of PIK3R2, AKT, can regulate the invasion, migration and division of cancer cells, including endometrial cancer, breast cancer and lung cancer cells (17,18). In addition, PIK3R2 encodes p85β, the expression of which was found to be upregulated in colon cancer (19). Therefore, it was hypothesized that YWHAB may regulate the malignant progression of colon cancer by binding to PIK3R2 protein.

The present study was designed to unravel the functional role of YWHAB in cell proliferation, cell cycle progression and apoptosis in colon cancer, in addition to examining its associated regulatory mechanism.

Materials and methods

Bioinformatics tools. GEPIA database version 2 (http://gepia.cancer-pku.cn/) analysis was used to examine YWHAB expression in colon cancer tissues. The information was obtained from The Cancer Genome Atlas normal and Genotype-Tissue Expression data (|Log2 fold change| cutoff, 1; q-value cutoff, 0.01) and analyzed using one-way ANOVA with Tukey's post hoc test. The 'COAD' dataset corresponding to colon cancer was used. In the Monarch Initiative database 2020 (https://monarchinitiative.org/), the term 'YWHAB' was searched and 560 interaction associations were obtained. Among the 560 interaction associations, a high throughput protein (PIK3R2) was identified based on affinity chromatography evidence analysis data included in the database.

Cell culture. The human intestinal epithelial cell line HIEC-6 and the DiFi colon cancer cell line were provided by BioVector NTCC, Inc., whilst human colon cancer cell lines HCT116, HCT8 and SW620 were obtained from American Type Culture Collection. All cell lines were incubated in DMEM (MilliporeSigma) at 37˚C with 5% CO_2. The medium was supplemented with 10% FBS (MilliporeSigma) and 1% penicillin/streptomycin (MilliporeSigma).

Cell transfection. HCT116 cells ($3x10^5$ cells/well) were inoculated onto six-well plates and cultured for 24 h at 37˚C with 5% CO_2. Short hairpin RNA (shRNA) sequences specific to YWHAB (shRNA-YWHAB-1; 5'-GGCTGAGCGATATGATGATAT-3' and shRNA-YWHAB-2, 5'-TGCAGCCTACACACCCAATTC-3') were inserted into the lentivirus expression plasmid pGCSIL-GFP, with shRNA-NC (sequence, 5'-TTCTCCGAACGTGTCACGT-3') as the corresponding negative control. The plasmids were obtained from Sangon Biotech Co., Ltd. pcDNA3.1 containing the PIK3R2 gene (Ov-PIK3R2) were utilized to overexpress PIK3R2 (accession no. NM_005027.4) whereas empty vector was used as the negative control (Ov-NC), which were provided by Shanghai GenePharma Co., Ltd. Following incubation, HCT116 cells were transfected with shRNA-NC (25 nM), shRNA-YWHAB-1/2 (25 nM), Ov-NC (50 nM) and Ov-PIK3R2 (50 nM), respectively, at 37˚C for 48 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). In another experiment, HCT116 cells were co-transfected with shRNA-YWHAB (25 nM) and Ov-PIK3R2 (50 nM) at 37˚C for 48 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the cells were harvested for other assays. Throughout all experiments, the cells in the Control group were un-transfected HCT116 cells.

Reverse transcription-quantitative PCR (RT-qPCR). After the isolation of total RNA from the indicated cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), cDNA was synthesized through reverse transcription using the iScript Reverse Transcription Supermix Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocols. Subsequently, SYBR® Premix Ex Taq™ reagent (Takara Bio, Inc.) was applied to perform qPCR on an ABI 7500 quantitative PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 10 min; followed by 40 cycles of 95˚C for 10 sec and 60˚C for 60 sec. The $2^{-\Delta\Delta Cq}$ method was applied to determine the relative gene expression with GAPDH serving as the endogenous reference (20). The following primer sequences were used: YWHAB forward, 5'-CATGAAGGCAGTCACAGAACA-3' and reverse, 5'-CTCACGGTACTCTTTGCCCAT-3'; PIK3R2 forward, 5'-AAAGGCGGGAACAATAAGCTG-3' and reverse, 5'-CAACGGAGCAGAAGGTGAGTG-3' and GAPDH forward, 5'- TGTGGGCATCAATGGATTTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'.

Western blotting. The concentration of proteins was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Inc.) after the extraction of total proteins from the indicated cells using RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.). Proteins (50 μg) were loaded per lane, separated by 8% SDS-PAGE and then transferred onto PVDF membranes. Subsequently, membranes were blocked with 5% skim milk for 1 h at room temperature, followed by the overnight incubation of membranes with primary antibodies against YWHAB (cat. no. ab32560; 1:1,000; Abcam), PIK3R2 (cat. no. ab180967; 1:2000; Abcam), p21 (cat. no. ab109520; 1:1,000; Abcam), cyclin D1 (cat. no. 26939-1-AP; 1:1,000; ProteinTech Group, Inc.), Bcl-2 (cat. no. ab32124; 1:1,000; Abcam), Bax (cat. no. ab32503; 1:1,000; Abcam), phosphorylated (p-) PI3K (cat. no. ab278545; 1:1,000; Abcam), PI3K (cat. no. ab32089; 1:1,000; Abcam), p-AKT (cat. no. ab81283; 1:5,000; Abcam), AKT (cat. no. ab8805; 1:500; Abcam) or

GAPDH (cat. no. ab9485; 1:2,500; Abcam) at 4˚C. GAPDH was used as a loading control for normalization. On the next day, the membranes were incubated with HRP-labeled Goat Anti-Rabbit secondary antibodies (cat. no. ab6721; 1:2,000; Abcam) for another 2 h at room temperature. Finally, the proteins were visualized using the ECL Detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.). ImageJ software (version 1.41; National Institutes of Health) was used to semi-quantify the bands of each protein.

Cell Counting Kit-8 (CCK-8) assay. HCT116 cells were seeded into 96-well plates at a density of $5x10^3$ cells and incubated for 24 h at 37˚C. Subsequently, 10 μl CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added into each well for further incubation at 37˚ with 5% CO_2 for 4 h. Finally, the absorbance was determined using a microplate reader (λ=450 nm).

Flow cytometry. A total of $1x10^6$ HCT116 cells were seeded into 24-well plates for incubation at 37˚C. When the cells grew to cover ~85% of the flask, they were harvested using trypsin without EDTA, centrifuged (5 min; 175 x g) at room temperature and washed twice with 3 ml PBS. Ethanol (70%) was used for cell fixation for 1 h at 4˚C. Cells were then centrifuged at 850 x g for 5 min at room temperature and washed twice with 3 ml PBS. Subsequently, 50 μg/ml PI (Thermo Fisher Scientific, Inc.) and 100 μg/ml RNase A (Thermo Fisher Scientific, Inc.) were added to the $1x10^6$ cells in a 100 μl cell suspension and incubated at 4˚C for 30 min. Finally, cell cycle was evaluated by flow cytometry (CytoFLEX; Beckman Coulter, Inc.) with the Flowjo 10 (FlowJo LLC) analysis software.

TUNEL. A Click-iT™ Plus TUNEL assay kit (cat. no. C10617; Invitrogen; Thermo Fisher Scientific, Inc.) was used to detect cell apoptosis using standard protocols. After rinsing with PBS three times, HCT116 cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.25% Triton-X 100 for 20 min at room temperature. TUNEL and FITC-deoxyuridine triphosphate solution (Roche Diagnostics GmbH) were added to the cells, which were incubated at 37˚C for 60 min in the dark. DAPI (0.5 μg/ml; Beijing Solarbio Science & Technology Co., Ltd.) was used to stain the nuclei for 5 min at room temperature and the cells were mounted in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). Finally, images of the positive apoptotic cells in a total of five fields of view per sample were captured using a fluorescence microscope (magnification, x200).

Co-immunoprecipitation (Co-IP) assay. Total proteins from HCT116 cells were isolated using the RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using BCA kits (Beyotime Institute of Biotechnology). The supernatant was centrifuged at 14,000 x g at 4˚C for 10 min to obtain whole-cell extracts. Subsequently, 400 μl whole-cell extracts ($2x10^6$ cells) were preincubated with 25 μg magnetic beads on a rotator for 2 h at 4˚C to clear non-specific bead binding. For immunoprecipitation, the extracts were incubated with 2 μg YWHAB (cat. no. A71754-050; 1:100; EpiGentek Group, Inc.) or PIK3R2 (cat. no. ab180967; 1:80; Abcam) antibodies overnight at 4˚C. Afterwards, 50 μg Protein G/A agarose beads (cat. no. 88803; Invitrogen; Thermo Fisher Scientific, Inc.) and 80 μl lysate were added, followed by incubation for 4 h at 4˚C. Following rinsing with PBS, the beads were resuspended in 5X SDS-PAGE loading buffer and boiled for 5 min at 100˚C to release the protein from the beads by centrifugation at 2,000 x g for 1 min at 4˚C. Western blotting was then performed on these immunoprecipitation products to detect PIK3R2 and YWHAB.

Statistical analysis. All experiments were repeated at least three times. All data collected from experiments are presented as the mean ± standard deviation and were analyzed using the GraphPad Prism 8.0 software (GraphPad Software, Inc.). One-way ANOVA with Tukey's post hoc test was applied to assess differences among different groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

YWHAB expression is increased in colon cancer cells but knocking it down inhibits cell proliferation. The GEPIA database revealed that the expression of YWHAB in colon cancer tissues was increased compared with that in normal tissues from healthy controls (Fig. 1A). YWHAB expression was examined at both mRNA and protein levels by RT-qPCR and western blotting, respectively. As shown in Fig. 1B and C, the mRNA and protein expression levels of YWHAB were markedly increased in the majority of the colon cancer cell lines tested compared with those in the HIEC-6 human intestinal epithelial cell line. In particular, HCT116 cells exhibited the highest expression levels of YWHAB among all colon cancer cell lines analyzed in the present study. Therefore, HCT116 cells were selected for subsequent experiments. To silence YWHAB expression, shRNA-YWHAB were transfected into HCT116 cells. RT-qPCR and western blotting were then performed to evaluate transfection efficacy. Compared with those in cells transfected with shRNA-NC, the mRNA and protein expression levels of YWHAB were significantly decreased in cells transfected with shRNA-YWHAB (Fig. 1D and E). shRNA-YWHAB-1 exhibited superior transfection efficacy, as demonstrated by markedly lower expression levels of YWHAB in HCT116 cells compared with those in the shRNA-YWHAB-2 group (Fig. 1D and E). The effect of YWHAB knockdown on the proliferation of HCT116 cells was next assessed using a CCK-8 assay. The results revealed that the proliferation of HCT116 cells was significantly reduced at 24, 48 and 72 h after transfection with shRNA-YWHAB compared with that in cells transfected with shRNA-NC (Fig. 1F).

YWHAB knockdown promotes cell cycle arrest in colon cancer cells at the G_0/G_1 phase. Compared with those in the shRNA-NC group, the numbers of cells in the G_0/G_1 phase were significantly elevated whilst the numbers of cells at the S and G_2/M phases were markedly reduced after knocking down YWHAB expression in HCT116 cells (Fig. 2A). Subsequently, western blotting was used to determine the expression levels of the G_1-S cell-cycle transition regulator cyclin D1 and G_1-checkpoint CDK inhibitor p21, where the results demonstrated that cyclin D1 protein expression

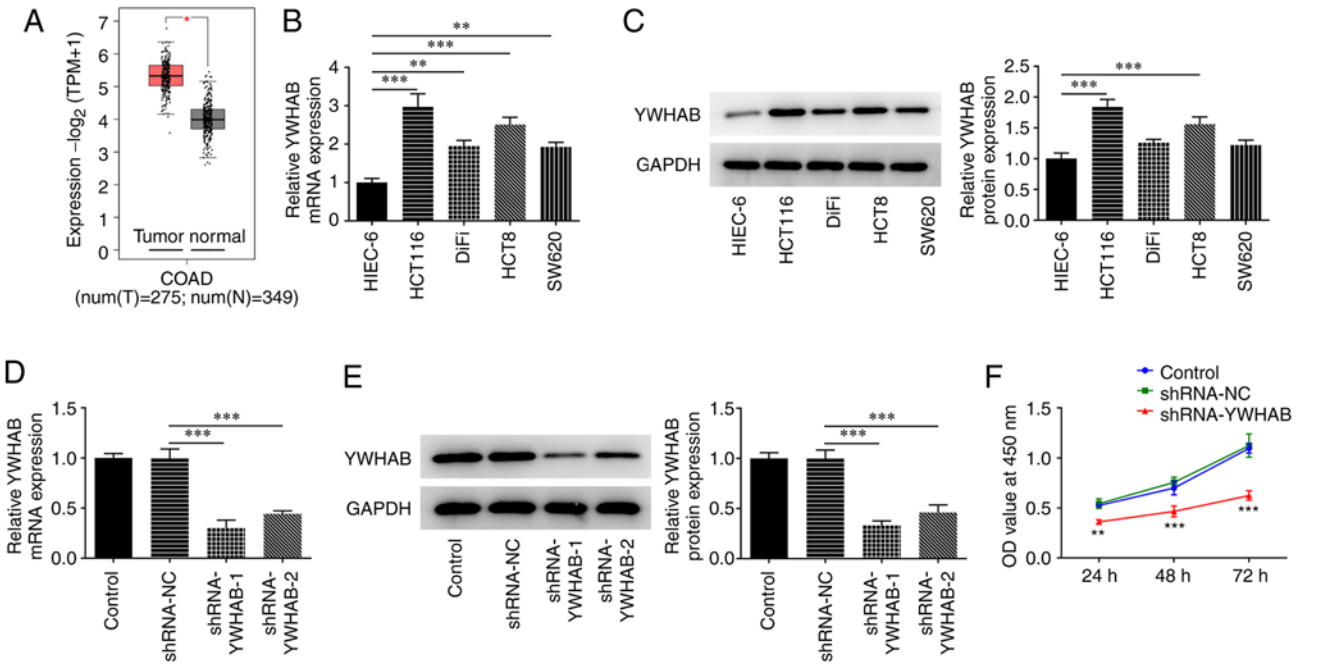

Figure 1. YWHAB expression is increased in colon cancer cells but knocking it down inhibits cell proliferation. (A) The expression of YWHAB in colon cancer tissues was predicted by the GEPIA database. The expression of YWHAB (B) mRNA and (C) protein was detected by RT-qPCR and western blotting, respectively. The expression of YWHAB (D) mRNA and (E) protein in transfected cells was detected by RT-qPCR and western blotting, respectively. (F) The cell proliferation was detected by Cell Counting Kit-8 assay. *P<0.05, **P<0.01 and ***P<0.001. COAD, colon adenocarcinoma; YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control; OD, optical density.

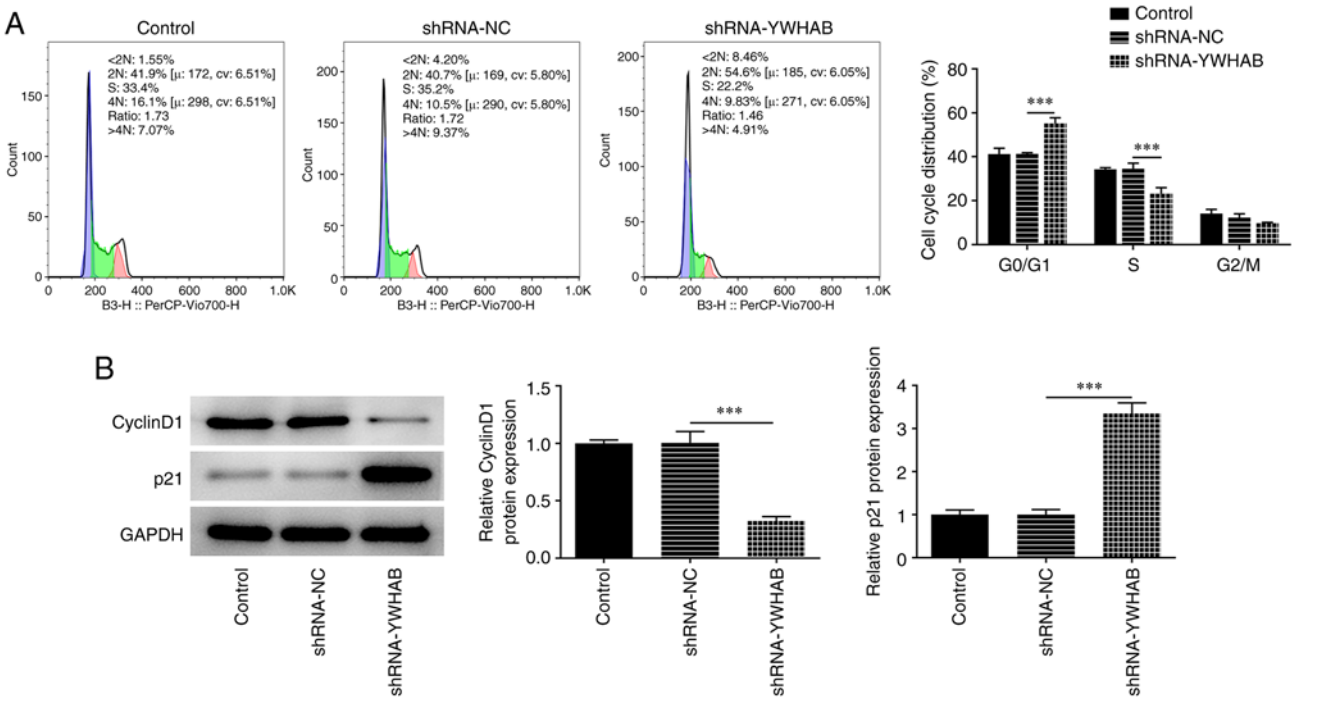

Figure 2. YWHAB knockdown promotes cell cycle arrest in colon cancer cells at the G_0/G_1 phase. (A) Cell cycle was detected by PI flow cytometry. (B) The expression of cell cycle markers cyclin D1 and p21 were detected by western blotting. ***P<0.001. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; shRNA, short hairpin RNA; NC, negative control.

was significantly decreased, while p21 protein expression was notably increased in the YWHAB knockdown group (Fig. 2B). These aforementioned results suggest that YWHAB knockdown can induce cell cycle arrest in colon cancer cells.

YWHAB knockdown promotes cell apoptosis. YWHAB knockdown significantly enhanced the apoptosis level of HCT116 cells compared with that in the shRNA-NC group, thus suggesting that YWHAB knockdown exerted apoptotic

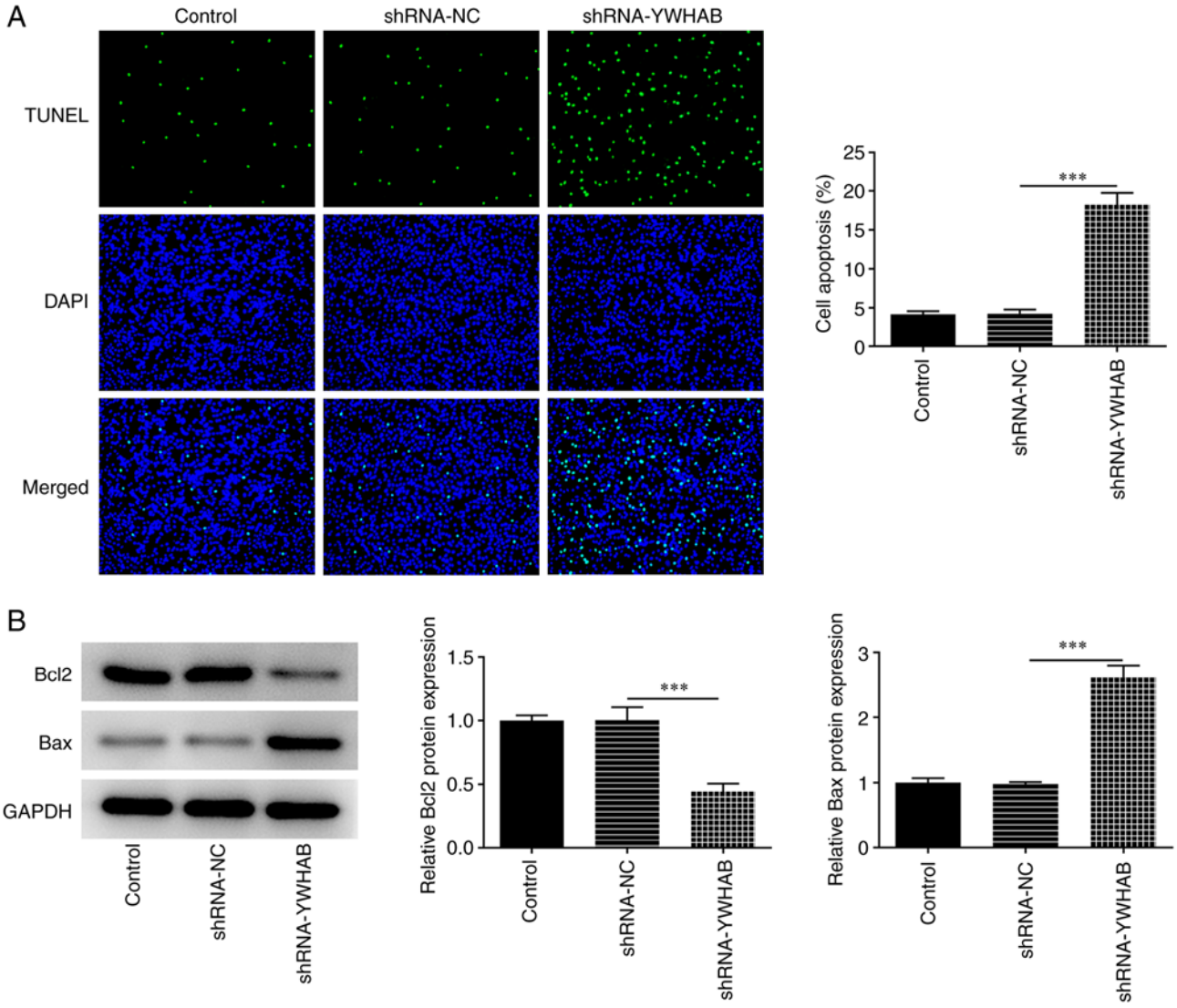

Figure 3. YWHAB knockdown promotes cell apoptosis. (A) Cell apoptosis was detected by TUNEL staining. (B) Expression of apoptosis regulator proteins was detected by western blotting. ***P<0.001. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; shRNA, short hairpin RNA; NC, negative control.

effects on HCT116 cells (Fig. 3A). Additionally, the expression of apoptosis regulators were determined by western blotting. The results revealed that Bcl2 expression was significantly decreased whilst Bax expression was significantly increased in HCT116 cells after transfection with shRNA-YWHAB compared with those in the shRNA-NC group (Fig. 3B).

YWHAB can bind to PIK3R2 in colon cancer cells. The Monarch Initiative database predicted that YWHAB can interact with PIK3R2 (Fig. 4A). The results in Fig. 4B suggested that the mRNA expression levels of PIK3R2 were increased in colon cancer cell lines compared with those in HIEC-6 cells. The results of Co-IP assay demonstrated that PIK3R2 was particularly enriched in lysate samples incubated with the anti-YWHAB antibody (Fig. 4C). In addition, the expression levels of PIK3R2 in HCT116 cells were significantly decreased after transfection with shRNA YWHAB, compared with those in the shRNA-NC group (Fig. 4D and E), suggesting that YWHAB positively regulated PIK3R2 expression in colon cancer.

YWHAB regulates cell proliferation and cell cycle arrest in colon cancer cells by binding to PIK3R2. To detect overexpression efficiency, RT-qPCR and western blotting were applied to examine the mRNA and protein expression levels of PIK3R2. As shown in Fig. 5A and B, PIK3R2 expression at both mRNA and protein levels was significantly enhanced after HCT116 cells were transfected with the Ov-PIK3R2 plasmids compared with that in the Ov-NC group. The reduction in the proliferation of YWHAB-silenced HCT116 cells was significantly reversed by PIK3R2 overexpression, suggesting that PIK3R2 overexpression exerted rescue effects on the proliferation of YWHAB-depleted HCT116 cells (Fig. 5C). Compared with that in the control group (shRNA-NC-transfected HCT116 cells), YWHAB downregulation increased the number of cells at the G_0/G_1 phase but decreased the number of cells in S phase, which was partially reversed after transfection with the Ov-PIK3R2 plasmids compared with those in the shRNA-YWHAB + Ov-NC group (Fig. 5D and E). Furthermore, YWHAB knockdown decreased cyclin D1 expression and increased p21 expression,

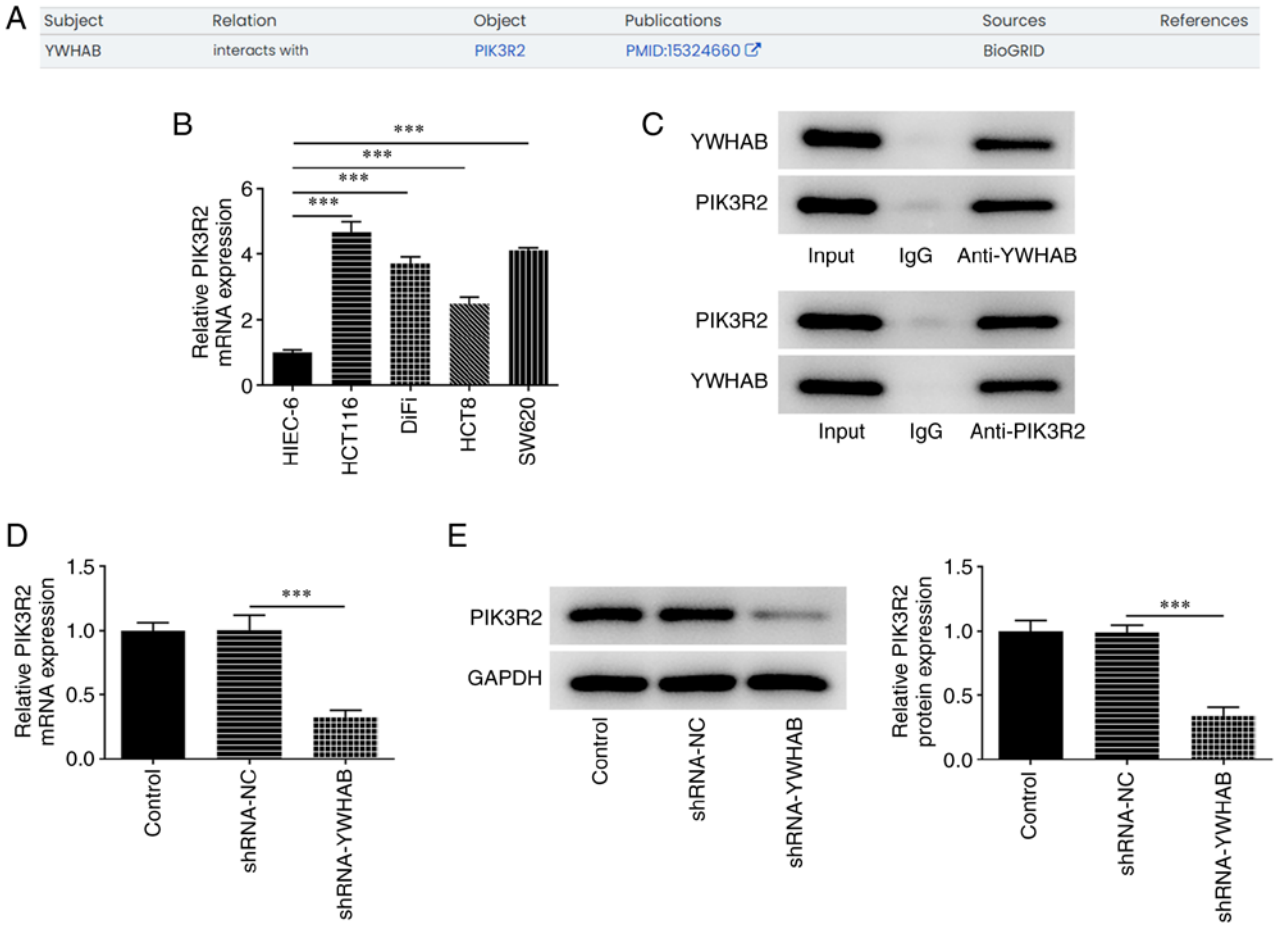

Figure 4. YWHAB can bind to PIK3R2 in colon cancer cells. (A) Monarch Initiative database predicted that YWHAB can bind to PIK3R2. (B) The mRNA expression of PIK3R2 was detected in colon cancer cell lines by RT-qPCR. (C) The binding of YWHAB and PIK3R2 was verified by co-immunoprecipitation assay. PIK3R2 (D) mRNA and (E) protein expression were detected by RT-qPCR and western blotting. ***P<0.001. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; PIK3R2, PI3K regulatory subunit 2; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control.

to both of which PIK3R2 overexpression exerted significant oppositive effects (Fig. 5F). Significantly increased cyclin D1 expression and decreased p21 expression were observed in the shRNA-YWHAB + Ov-PIK3R2 group compared with those in the shRNA-YWHAB + Ov-NC group (Fig. 5F). These aforementioned results suggest that YWHAB regulates cell proliferation and cell cycle arrest in colon cancer cells by binding to PIK3R2.

YWHAB regulates apoptosis in colon cancer cells by binding to PIK3R2. Compared with that of cells in the control group (shRNA-NC-transfected HCT116 cells), the apoptosis of HCT116 cells was significantly promoted by YWHAB knockdown, which was then partially but significantly reversed by the overexpression of PIK3R2 (Fig. 6A). In addition, YWHAB knockdown decreased Bcl2 expression but enhanced Bax expression compared with those in the control group (shRNA-NC-transfected HCT116 cells) (Fig. 6B). However, the effects of YWHAB knockdown on the expression of these apoptosis regulators were significantly reversed by PIK3R2 overexpression. Significantly increased Bcl2 expression and decreased Bax expression were both observed in the shRNA-YWHAB + Ov-PIK3R2 group compared with those in the shRNA-YWHAB + Ov-NC group (Fig. 6B). Therefore, these aforementioned results suggest that YWHAB regulates apoptosis in colon cancer cells by binding to PIK3R2.

YWHAB regulates the PI3K/AKT signaling pathway by binding to PIK3R2. As one of the PI3K p85 subunit family members, PIK3R2 is a key gene in the PI3K/AKT signaling pathway (15-18). To investigate the effect of YWHAB on the PI3K/AKT signaling pathway, western blotting was performed to examine the protein levels of PI3K/AKT signaling pathway markers p-PI3K, PI3K, p-AKT and AKT. As shown in Fig. 7, YWHAB knockdown significantly decreased the levels of p-PI3K and p-AKT compared with those in the control group (shRNA-NC-transfected HCT116 cells), whilst PIK3R2 overexpression significantly reversed these aforementioned effects. Ov-PIK3R2 transfection significantly increased the levels of p-PI3K and p-AKT in the shRNA-YWHAB + group compared with those in the shRNA-YWHAB + Ov-NC group. These results suggest that YWHAB can regulate the PI3K/AKT signaling pathway, likely by targeting PIK3R2.

Discussion

In the present study, the Monarch Initiative database analysis predicted that YWHAB could bind to PIK3R2. In the present

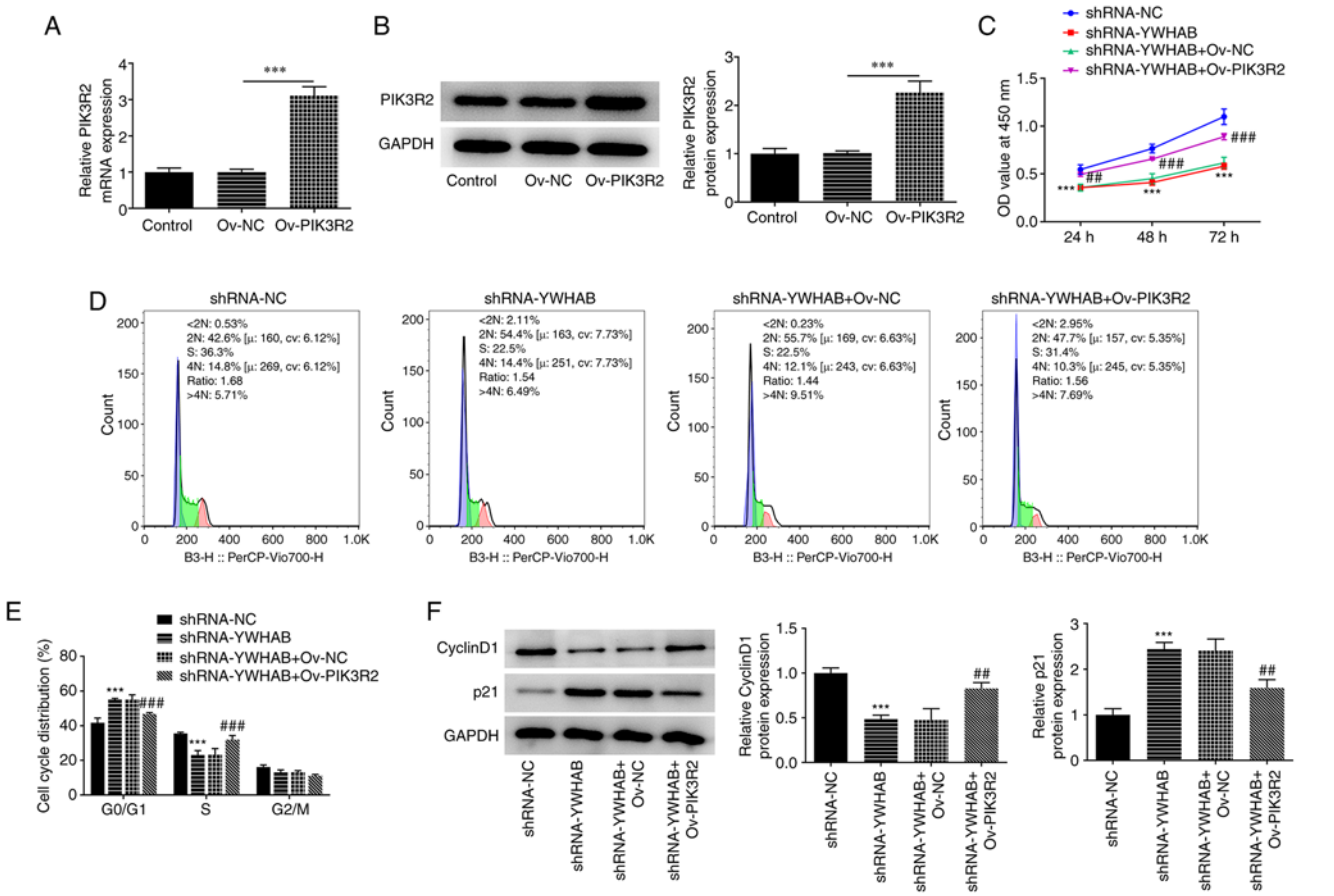

Figure 5. YWHAB regulates cell proliferation and cell cycle arrest in colon cancer cells by interacting with PIK3R2. The expression of PIK3R2 (A) mRNA and (B) protein in transfected cells was detected by reverse transcription-quantitative PCR and western blotting. ***P<0.001. Un-transfected HCT116 cells were used as the Control. (C) Cell proliferation was detected by Cell Counting Kit-8 assay. (D) Cell cycle progression was detected by flow cytometry and (E) quantified. (F) The expression of cell cycle markers was detected by western blotting. ***P<0.001 vs. shRNA-NC; ##P<0.01 and ###P<0.001 vs. shRNA-YWHAB + Ov-NC. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; PIK3R2, PI3K regulatory subunit 2; shRNA, short hairpin RNA; NC, negative control; Ov, overexpressing vector.

study, the cellular mechanism in colon cancer was examined, revealing that YWHAB knockdown suppressed proliferation, induced cell cycle arrest and promoted apoptosis in HCT116 cells. Additionally, further experiments demonstrated that YWHAB regulated cell proliferation, the cell cycle and apoptosis in colon cancer and the PI3K/AKT signaling pathway, by binding to PIK3R2.

Colon cancer has attracted the attention of research communities worldwide due to its high rates of mortality (21). Aberrant cell proliferation is a typical feature of malignant tumors, such that inhibition of proliferation has been found to suppress tumor development (22). It has been previously acknowledged that apoptosis forms an important part of the innate tumor-suppression mechanism, which has been considered to be a promising target for cancer therapy (23,24). Cell cycle dysregulation is another frequently reported feature of cancer in humans and several therapeutic strategies have been investigated to target the cell division cycle in cancer (25,26). Furthermore, the availability of molecular markers greatly facilitated the diagnosis and prognosis of colon cancer. In particular, molecular targeted therapies are becoming important for the treatment of colon cancer (27,28). YWHAB is a member of the 14-3-3 protein family and has been reported to regulate the physiology of numerous cancer types, including lung cancer, prostate cancer and hepatocellular carcinoma (29-31), suggesting that YWHAB may serve a carcinogenic role in various organs. Additionally, YWHAB expression was demonstrated to be upregulated in colon cancer cells, where its upregulation was associated with poorer prognosis in patients with this disease (14). Consistent with these findings, the mRNA and protein expression levels of YWHAB were found to be markedly enhanced in colon cancer cells in the present study. However, this upregulated YWHAB expression was decreased after the cells were transfected with shRNA-YWHAB. In addition, YWHAB knockdown induced cycle arrest in colon cancer cells at the G_0/G_1 phase, as demonstrated by the increased expression levels of p21. p21 is a tumor suppressor that has been documented to be involved in the regulation of cycle arrest and cell proliferation during G_0/G_1 phase (32). In cell cycle progression, p21 may inhibit the CDK activity to block the entry into S phase when DNA damage occurs in G_1 phase (33). Knocking down YWHAB expression also promoted the apoptosis of colon cancer cells and increased the expression levels Bax, a pro-apoptotic protein.

PIK3R2 encodes the p85β regulatory subunit of PI3K, the expression of which has been frequently found to be increased in cancer (34). PIK3R2 has been reported

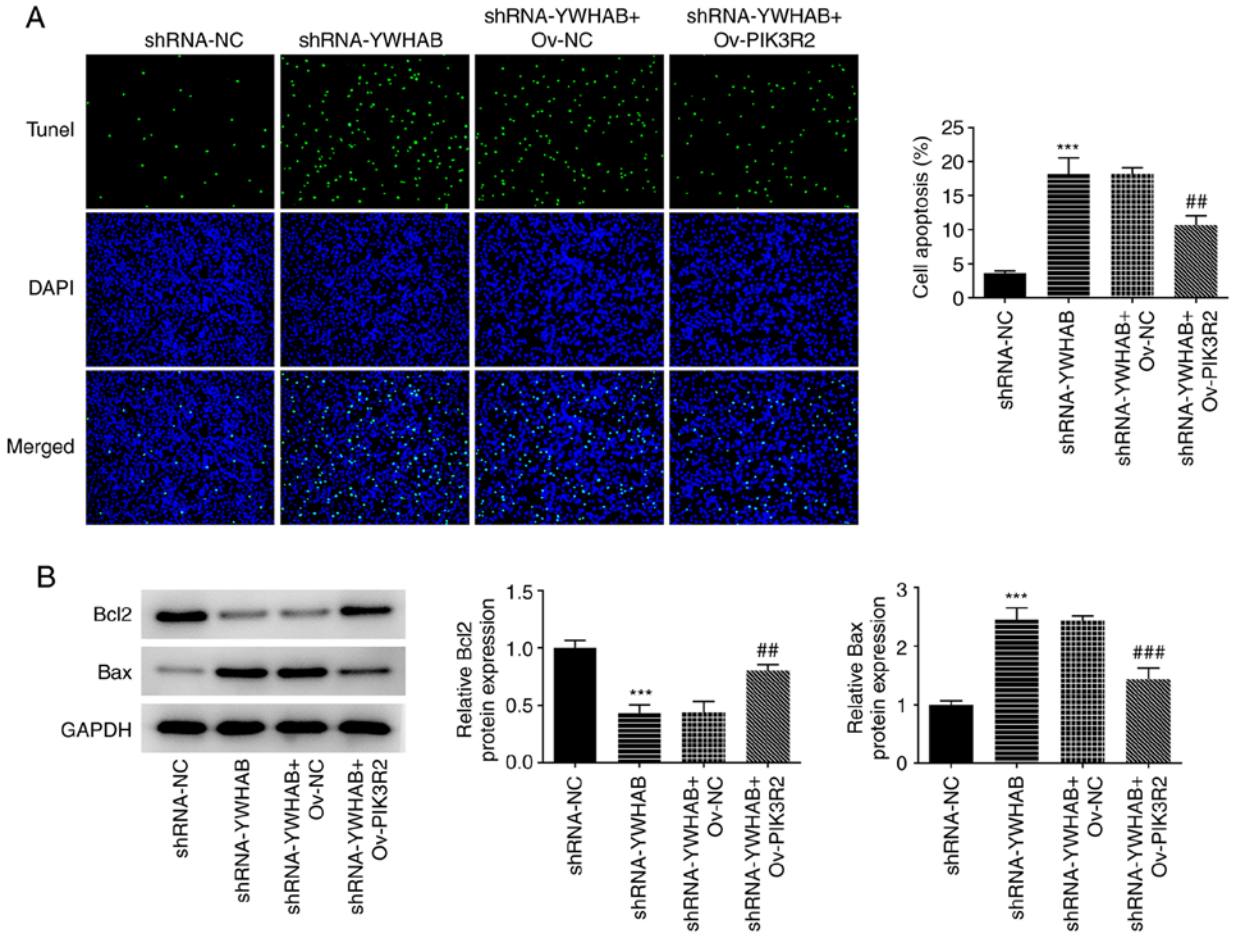

Figure 6. YWHAB regulates colon cancer cell apoptosis by binding to PIK3R2. (A) Cell apoptosis was detected using TUNEL staining. (B) The expression of apoptosis regulators was detected by western blotting. ***P<0.001 vs. Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. shRNA-YWHAB + Ov-NC. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; PIK3R2, PI3K regulatory subunit 2; shRNA, short hairpin RNA; NC, negative control; Ov, overexpressing vector.

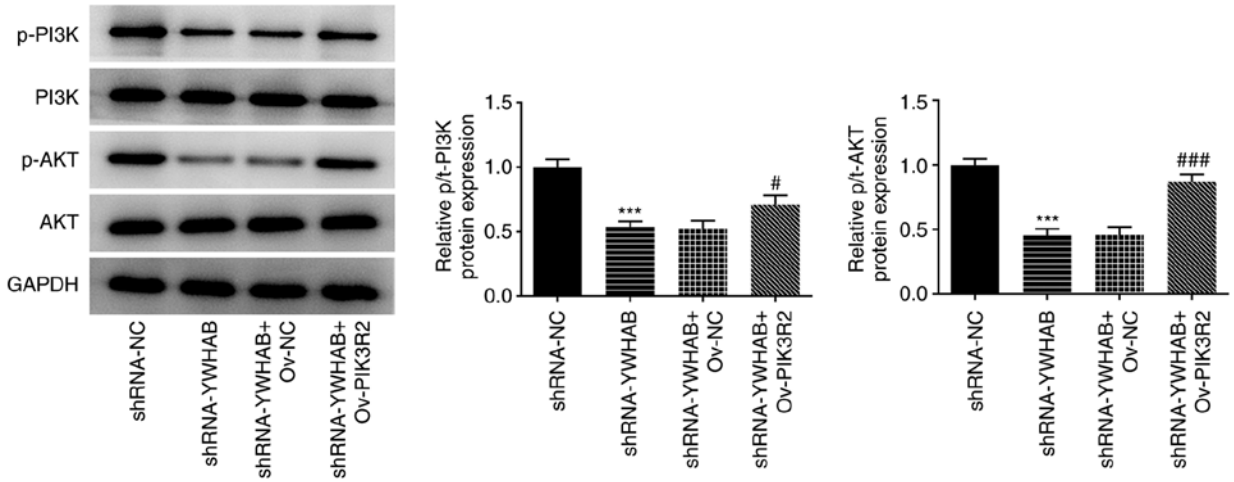

Figure 7. YWHAB regulates the PI3K/AKT signaling pathway by binding to PIK3R2. The expression of PI3K/AKT signaling marker proteins was detected by western blotting. ***P<0.001 vs. Control; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. shRNA-YWHAB + Ov-NC. YWHAB, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β; PIK3R2, PI3K regulatory subunit 2; shRNA, short hairpin RNA; NC, negative control; Ov, overexpressing vector; p-, phosphorylated; t-, total.

to regulate the progression of numerous cancer types. MicroRNA-126 has been found to exert suppressive effects on the proliferation and metastasis of prostate cancer by targeting of PIK3R2 (35). In addition, ephrin A4 was previously observed to promote proliferation and tumor metastasis through PIK3R2 in hepatocellular carcinoma (36). In the

present study, it was revealed that PIK3R2 expression was markedly upregulated in colon cancer cells. According to the Monarch Initiative database, YWHAB could interact with PIK3R2, which was verified by Co-IP assay in the present study. To further explore the mechanism of YWHAB in colon cancer, additional functional experiments were performed. YWHAB knockdown was found to inhibit cell proliferation, induce cycle arrest and promote apoptosis in HCT116 cells. In addition, all of the aforementioned effects induced by YWHAB knockdown were reversed by PIK3R2 overexpression. This suggests that YWHAB can regulate cell proliferation, cell cycle arrest and apoptosis in colon cancer by binding to PIK3R2.

The aberrant activation of the PI3K/AKT signaling pathway, which consists of PI3K and their downstream mediator AKTs, is associated with cell proliferation (37,38). Being a member of the PI3K p85 subunit family, PIK3R2 has been demonstrated as a core regulator in the activation of the PI3K/AKT signaling pathway (39). In the present study, the protein levels of p-PI3K and p-AKT were decreased by YWHAB knockdown compared with those in cells transfected with shRNA-NC. By contrast, PIK3R2 overexpression reversed these aforementioned effects, suggesting that YWHAB regulated the PI3K/AKT signaling pathway by binding to PIK3R2.

In conclusion, the present study first revealed the role of YWHAB in the malignant progression of colon cancer cells and demonstrated that YWHAB regulated PI3K/AKT signaling by binding to PIK3R2, which was the novelty of the present study. The present findings could guide the future research and development of target drugs. However, limitations exist in the present study. Flow cytometry analysis was not performed for apoptosis analysis. In addition, clinical tissue analysis, the role of YWHAB in other colon cancer cells and an animal model of colon cancer were not explored. Therefore, further studies are required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TZ and XZ conceived and designed this study. XZ conducted the experiments and AC analyzed the experimental data. All authors have read and approved the final manuscript. TZ and XZ confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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