Original Article

YWHAE affects proliferation, migration and apoptosis of colorectal cancer by regulating extracellular vesicles secretion and Wnt/β-catenin signaling pathway

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Background: Colorectal cancer (CRC) has higher rates of metastasis, recurrence, and poor clinical prognosis. The 14-3-3ε (YWHAE) protein is closely related to the occurrence and development of CRC. Here, we aimed to explore the effects of YWHAE on the proliferation, migration, and apoptosis of CRC cells, and elucidate its mechanism.

Methods: Western blot and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were used to detect YWHAE protein and messenger RNA (mRNA) expression levels in CRC cell lines. Cell viability was detected by Cell Counting Kit-8 (CCK-8) method. The cell proliferation activity was detected by 5-ethynyl-2'-deoxyuridine (EdU) assay. The effect of cell migration was detected by scratch healing test. The number of cell migration was detected by Transwell assay. The apoptosis of cells in each group was detected by flow cytometry. The effect of altered YWHAE expression on extracellular vesicles (EVs) secretion was detected by nanoparticle tracking analysis (NTA) and western blot.

Results: In this study, the results of reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot showed that YWHAE was highly expressed in CRC cells and tissues. Moreover, we constructed expression silenced cells and overexpression cells. Consistently, CCK-8, EdU assay, scratch healing test and Transwell assay showed that the silenced expression of YWHAE inhibited migration, and proliferation, while flow cytometry analysis promoted the apoptosis in YWHAE silenced cells. Mechanically, the expression levels of Wingless-related integration site (Wnt)/β-catenin and related genes (E-cadherin, cyclin D, c-myc, vimentin, P-120 catenin) in the silenced group signaling pathway were partially decreased. The results of NTA and western blot suggested that the ability of expression silenced cells to secrete EVs was weakened. In addition, the expression level of β-catenin in EVs from silenced cells was significantly decreased, which inhibited the proliferation activity of tumor microenvironment (TME) cells. Nevertheless, the data of overexpression group showed the opposite trend.

Conclusions: Altogether, these results demonstrate that silencing YWHAE expression can inhibit the proliferation and migration of CRC cells and promote cell apoptosis, which may be related to the inhibition of Wnt/β-catenin signaling and EVs secretion.

Keywords: Colorectal cancer (CRC); YWHAE; extracellular vesicles secretion (EVs secretion); Wnt/β-catenin signaling pathway

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Introduction

Colorectal cancer (CRC) is the third most common malignant tumor worldwide and the second leading cause of cancer-related death (1). Although CRC screening has become more widely available and its overall incidence and deaths have declined, about a quarter of CRC patients have metastases at diagnosis, and nearly half eventually develop metastases (2). At present, there are many therapeutic methods for CRC, such as surgery, radiotherapy, chemotherapy and immunotherapy. However, patients diagnosed with CRC at a later stage have higher rates of metastasis and recurrence, and the clinical prognosis is still poor (3). Therefore, it is of great significance to explore the molecular mechanism of CRC occurrence and development and to find new biological targets conducive to early diagnosis and treatment for improving the survival rate of patients.

The 14-3-3 protein family is common in various eukaryotes and consists of seven subtypes (β, ε, η, γ, τ, ζ, and σ) (4). It is evolutionarily conserved and is one of the most abundant proteins in mammalian cells (5). YWHAE, also known as14-3-3ε, is encoded by the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon gene and is one of the important members of the family (6). It is involved in biological processes such as cell adhesion, cell cycle, tumor drug resistance, signal transduction and neurodevelopment, which is closely related to many diseases. In CRC, it has been reported that NKD1 activates the transcriptional activity of *YWHAE* gene to promote glucose uptake (7). YWHAE has also been reported to be a regulation factor of autophagy and promotes the mechanism of nuclear export of heterogeneous nuclear ribonucleoprotein C (hnRNPC) in CRC (8). Moreover, YWHAE lncRNA can compete for endogenous RNA through direct interaction with miR-323a-3p and miR-532-5p, thus up-regulating K-RAS/ERK/1/2 (extracellular regulated protein kinases) and PI3K-AKT (phosphatidylinositide 3-kinases-protein kinase B) signaling pathways and promoting the cell cycle progression of CRC (9,10). In gastric cancer cell lines, *YWHAE* acts as a negative regulator of *MYC* and *CDC25B*, MYC induces the cell proliferation, invasion and migration through the induction of CDC25B and the reduction of YWHAE (11). In breast cancer, YWHAE overexpression significantly increases the progression and chemoresistance (12). In kidney cancer, ANKZF1 promotes tumor progression and lymphangiogenesis by attenuating YWHAE-driven cytoplasmic retention of YAP1 (13). In ovarian cancer, YWHAE can influence the malignant behaviour by regulating the PI3K/AKT and MAPK pathways, representing a risk factor for the prognosis that is positively correlated with HE4 expression (14). However, the effects of YWHAE on tumorigenesis and its mechanism remain to be further explored.

Extracellular vesicles (EVs) are a class of bilayer membrane vesicles secreted by all cells under physiological and pathological conditions. EVs can carry a variety of bioactive substances, including proteins, lipids, messenger RNA (mRNA), microRNA (miRNA) and metabolites, playing a very important role in cell information exchange (15). In recent years, EVs has gradually become a hot topic in medical research. In liquid tumor biopsy, EVs has become a new type of phenotypic regulator of cancer cells, and EVs

Highlight box

Key findings

- This finding found that YWHAE was highly expressed in colorectal cancer (CRC) cells and tissues. Furthermore, the 14-3-3ε (YWHAE) regulates extracellular vesicles (EVs) secretion and Wingless-related integration site (Wnt)/β-catenin signaling pathways, providing a new potential target for molecular targeted therapy.

What is known and what is new?

- YWHAE is involved in biological processes such as cell adhesion, cell cycle, tumor drug resistance, signal transduction and neurodevelopment, which is closely related to many diseases.
- The specific mechanism by which YWHAE effects CRC by regulating EVs secretion and the Wnt/β-catenin signaling pathway is unclear.

What is the implication, and what should change now?

- Silencing YWHAE expression can inhibit the proliferation and migration of CRC and promote apoptosis, which may be related to reducing the expression of related genes in Wnt/β-catenin signaling pathway and inhibiting the secretion of EVs in tumor cells.

can also be used as a carrier of drug therapy (16), which is expected to be a new non-invasive biomarker in molecular detection (17), and has a good prospect of clinical diagnosis, treatment and prognosis in CRC (18).

Wingless-related integration site (Wnt)/β-catenin signaling pathway is considered to be the central mechanism of CRC occurrence and a key therapeutic target for CRC (19). However, the regulatory mechanism of this signaling pathway is complex and is not well understood despite extensive research. Whether YWHAE effects CRC by regulating the Wnt/β-catenin signaling pathway is unclear. Therefore, this study investigated the effects of YWHAE on the growth, proliferation, migration and apoptosis of CRC, and discussed the mechanism of action, laying a foundation for elucidation of the molecular mechanism of *YWHAE* gene and the development of CRC as well as clinical targeted therapy. We present this article in accordance with the MDAR reporting checklist (available at https://amegroups.com/article/view/10.21037/tcr-24-1910/rc).

Methods

Cell lines and cell culture

The human cervical cancer cell line HeLa (SCSP-504), the human embryonic kidney cell line HEK293T (SCSP-502), the human adenocarcinomic alveolar basal epithelial cell line A549 (SCSP-503), the CRC cell lines CaCO2 (SCSP-5027), HT29 (SCSP-5032), HCT116 (SCSP-5076), SW480 (SCSP-5033), SW620 (TCHu101), NCM460, and the human bronchial epithelial cell line HBE (CC2541) were stored in our laboratory. They were purchased from Shanghai Institute of Biology, Chinese Academy of Sciences (Shanghai, China). All cell lines were subjected to Short Tandem Repeats (STR) profiling and mycoplasma contamination detection. Cells were cultured in high glucose (4.5 g/L) Dulbecco's modified Eagle medium (DMEM) (C11995500BT, Gibco, MA, USA) supplemented with 10% fetal bovine serum (FBS) (10099141, Gibco, MA, USA) and 1% penicillin-streptomycin (15070063, Thermo Fisher Scientific, MA, USA) in a 37 ℃ humidity-controlled incubator with 5.0% CO_2.

Data collection

To investigate YWHAE expression levels in CRC patient, 51 normal (including 41 colon and 10 rectum) and 644 tumor samples (including 478 colon and 166 rectum samples) were downloaded from The Cancer Genome Atlas (TCGA) database (http://cancergenome.nih.gov). TCGA is open-ended and public and does not need the approval of a local ethics committee. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Generation of YWHAE silenced or overexpressed plasmids and stable cell lines

Sh-YWHAE oligo fragments sequences (forward primers: 5'-ccggcgctgagtgaagaaagctatactcgagtatagctttcttcactcagcgtttttg-3', reverse primer: 5'-aattcaaaaacgctgagtgaagaaagctatactcgagtatagctttcttcactcagcg-3') were synthesized by Genescript (Nanjing, China) and cloned into pLKO.1 plasmid (Addgene, MA, USA) using the restriction sites AgeI and EcoRI. YWHAE target fragment was amplified from SW620 cell genome, forward primer: 5'-cgacgcgtatggatgatcgagaggatct-3', reverse primer: 5'-ggactagttcacttatcgtcatcgtctttgtaatcctgattttcgtcttccacgt-3'. The oligo fragments were synthesized by Genescript. The plasmid psin-Puro (Addgene) was cut with the enzymes MluI and SpeI, and the YWHAE target fragment and Flag fragment were connected to the vector psin-Puro by homologous recombination using ClonExpress MultiS One Step Cloning Kit (C113-02, Vazyme, Nanjing, China). After sequencing, the psin-YWHAE + Flag plasmid was constructed successfully.

plko.1-sh-YWHAE and psin-YWHAE + Flag plasmids were mixed with psPAX2 and pMD2G plasmids respectively with Lipofectamine 200 (11668-019, Thermo Fisher Scientific, MA, USA) at a 10:5:1 molar ratio. They were dissolved in Opti-MEM, incubated at room temperature for 15 min, and transfected into 70–80% density HEK 293T cells. After 6 hours, the medium was changed and 2% FBS was added. After 48 hours, lentiviral particles were collected from the supernatant of the medium and filtered with a 0.45 μm filter. The virus particles were stored in the refrigerator at −80 ℃. SW620 cells were inoculated in 6-well plates, infected with plko.1-sh-YWHAE and psin-YWHAE + Flag lentivirus suspension, and added with 4 μg/mL of polybrene. After 48 hours of infection, 1 μg/mL of puromycin was added for drug screening. Stable expression cell lines were obtained after 2 weeks of drug screening.

Cell Counting Kit-8 (CCK-8) assay

Cells in the silenced and overexpressed groups were

inoculated into 96-well plates according to the density of 6×10^3 cells/well, and 100 µL CCK-8 reagent (A311-02, Vazyme, Nanjing, China) was prepared with serum-free DMEM after culture for 12, 24, 36, 48, and 60 hours, respectively, and incubated for 2 hours away from light. The optical density (OD) values of each hole were measured at 450 nm wavelength using an enzyme-labeled instrument.

5-Ethynyl-2'-deoxyuridine (EdU) assay

Cells (2×10^5) were inoculated into the 12-well culture plate, the culture medium was removed, and EdU treatment was carried out according to the instructions of the EdU test kit (C10310-1, RiboBio, Guangzhou, China). The percentage of EdU positive stained cells (red) in the total cells (blue) indicated the cell proliferation rate.

Transwell assay

The Transwell chamber was placed into a 24-well plate, and the cells of the above subgroups were added to the upper chamber according to 5×10^4 cells, and cultured for 24 hours. The chamber was removed and fixed with 4% paraformaldehyde for 20 min, and 0.1% crystal violet (C0121, Solaibao Technology, Beijing, China) staining solution was added. After rinsing and drying, they were photographed with a microscope, and the average number of cells in each field of view was calculated as the number of migrated cells.

Scratch healing test

The cells were inoculated on 6-well plates and cultured until the cell density reached 80%. After that, the cells were marked with medium gun tip, and the exfoliated cell residue was cleaned with phosphate buffered saline (PBS) buffer. The cells were continued to be cultured for 24 hours under normal conditions, photographs were taken under microscope, and the scratch boundary distance between 0 and 24 hours cells was analyzed using Image proPlus software. Mobility (%) = (0 h scratch spacing – 24 h scratch spacing)/0 h scratch spacing ×100%.

Flow cytometry

After digestion with pancreatic enzymes, cells were collected and washed twice with cold PBS, and the cell density was adjusted to 1×10^6 cells /mL. The procedure was carried out in accordance with the Annexin V/propidium iodide (PI) apoptosis Assay Kit (abs50001b, Universal Biotech, Shanghai, China) and the apoptosis rate was detected using flow cytometry (Biosciences, New Jersey, USA). The experiment was repeated for 3 times, and the apoptosis rate of labeled cells in each group was measured and calculated.

EVs extraction

EVs were isolated using either ultracentrifugation as previously described or Total Exosome Isolation Kit (4478359, Thermo Fisher Scientific) accordingly. For ultracentrifugation, the supernatant medium derived from 5×10^7 cells was harvested. The cells were cultured in MV-free FBS medium for 24 hours and was collected. Centrifuge at 300 ×g at 4 ℃ for 10 min, discard the precipitation. Centrifuge at 4 ℃ at 3,000 ×g for 30 min, transfer the supernatant to a new centrifuge tube. Then, centrifuge at 4 ℃ at 10,000 ×g for 30 min. After filtration by a 0.22 µm filter, the culture medium was transferred to ultracentrifugal tubes, centrifuged at 4 ℃ and 120,000 ×g for 70 min using Beckman 70Ti rotor (Beckman, Bremen, Germany). The EVs obtained by ultracentrifugation was dissolved with 1× PBS and could be temporarily kept in the refrigerator at 4 ℃. For using isolation kit, the cell was harvested and centrifuged 5 min at 300 ×g, 30 min at 3,000 ×g, 30 min at 10,000 ×g to remove the cells and debris. The cell-free medium was added the EVs isolation reagent according to the instructions, and left for overnight at 4 ℃. After centrifugation at 10,000 ×g for 1 hours at 4 °C, the EVs pellets were resuspended in 1× PBS and either used or kept at –80 °C for long-term storage.

Nanoparticle tracking analysis (NTA)

The collected EVs were diluted and filtered with a 0.22 µm filter. The sample is manually injected into the sample chamber at ambient temperature. The lens of each sample was set to 10, the acquisition time was set to 20 s, the detection threshold was set to 7, and the measurements were made 3 times. Each video analyzes at least 200 complete tracks. Capture and analyze data with the NTA analysis software version 3.2.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA from cultured cells and tissues were extracted

Table 1 Sequences of the primers for RT-qPCR

Name	Direction	Primer sequence
18S	PF	GTAACCCGTTGAACCCCATT
	PR	CCATCCAATCGGTAGTAGCG
YWHAE	PF	AATGACAGAACTTCCACCAAC
	PR	TCTTCACTCAGCGTATCCA
β-catenin	PF	AAGTGGGTGGTATAGAGGCTCTTG
	PR	GATGGCAGGCTCAGTGATGTC
E-cadherin	PF	GAGAGGAATCCAAAGCCTCAGGTC
	PR	CTGGTTATCCATGAGCTTGAGATTG
c-Myc	PF	GGCTCCTGGCAAAAGGTCA
	PR	CTGCGTAGTTGTGCTGATGT
P120-catenin	PF	TAATGAAGACTGTAAGCCACG
	PR	CTGAACAATGAAAATGAGGG
Cyclin D	PF	CCGTGAAAAAGAGCCGCCTG
	PR	CGGATGGAGTTGTCGGTGTA
Vimentin	PF	CCTTGAACGCAAAGTGGAATC
	PR	AGGTCAGGCTTGGAAACATC

PF, forward primer; PR, reverse primer; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

using TRIzol reagent (T9424, Sigma, Germany) according to manufacturer's instructions, and 1 μg total RNA was reverse-transcribed into complementary DNA (cDNA) using PrimeScript™ reverse transcription kit (2621, TaKaRa, Dalian, China). The upstream and downstream primer sequences of each gene were shown in *Table 1*. The reaction system was prepared according to the kit instructions, and the PCR reaction was performed with Evagreen Dye (31000-T, Biotium, USA). Reaction conditions: 95 ℃, 15 s; 60 ℃, 30 s; 72 ℃, 30 s (40 cycles). The mRNA internal reference in the amplified cells is 18S RNA or GAPDH, and the expression relative to the internal reference can be expressed by $2^{-\Delta Ct}$, where ΔCt = Ct sample – Ct internal reference.

Western blot

Cells or EVs were lysed in RIPA buffer (P0013B, Beyotime, Shanghai, China) on ice. Lysates were centrifuged for 10 min with 12,000 ×g at 4 ℃, and the protein concentration was determined by BCA Protein Quantification Kit (E112-02, Vazyme, Nanjing, China). Then protein lysates were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, followed by transferred to polyvinylidene fluoride membrane. Next, the membrane was blocked using specific antibodies and incubated with SuperPico ECL Chemiluminescence Kit (E422-02, Vazyme, Nanjing, China). The bands were finally detected using chemiluminescence imaging system (Tanon, Shanghai, China) and analysed using ImageJ software. GADPH, β-actin or α-tubulin was used as internal control. The primary antibodies against CD63 (25682-1-AP, 1:1,000), ALIX (12422-1-AP, 1:1,000) and TSG101 (14497-1-AP, 1:1,000) were purchased from Proteintech (IL, USA); antibodies against Flag (14793, 1:1,000), β-actin (4970, 1:1,000) were purchased from Cell Signalling Technology (MA, USA); antibody against CD9 (ab92726, 1:1,000) was purchased from Abcam (Cambridge, UK); antibody against GADPH (sc-47724, 1:1,000), YWHAE (sc-135816, 1:1,000), β-catenin (sc-393501, 1:1,000), α-tubulin (Cat. # sc-53646, 1:1,000) were purchased from Santa Cruz Biotechnology (CA, USA).

Statistical analysis

All experiments were independently repeated at least 3 times. The experimental data were processed by GraphPad Prism 8.0 software, the results were expressed as mean ± standard error of the mean (SEM), and the data were compared and analyzed by Student's *t*-test. The experiments were analyzed by one-way analysis of variance (ANOVA) and then analyzed by Tukey's test. P<0.05 (*): statistical difference; P<0.01 (**): significant difference; P<0.001 (***): very significant difference.

Results

Expression levels of YWHAE in different cell lines and CRC tissues

Firstly, several common cell lines (HEK293T, A549, HeLa, CACO2, HT29, HCT116, SW480, SW620, NCM460) were selected to extract the proteins and RNA. Western blot and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were used to analyze the expression levels of YWHAE protein (*Figure 1A* and Figure S1A) and mRNA (*Figure 1B*) in various cells. The results showed that the expression levels of YWHAE mRNA and protein were higher in CRC cell lines, with the highest levels observed

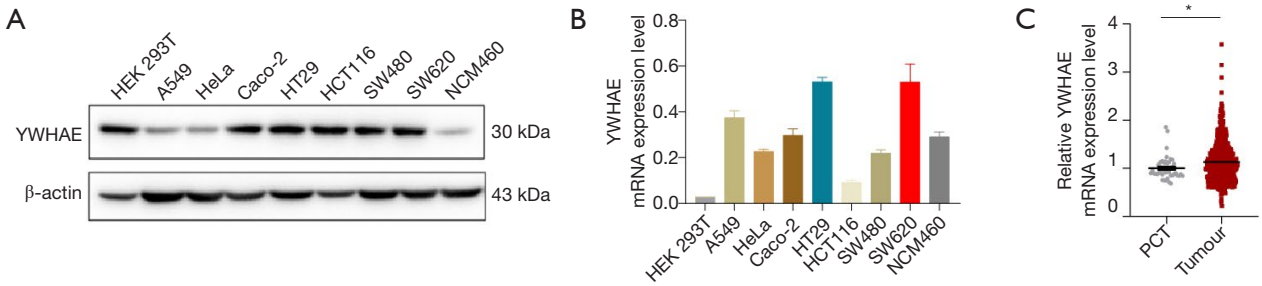

Figure 1 The expression levels of YWHAE in different cell lines and CRC tissues. (A) The YWHAE protein expression levels in different cells. (B) The YWHAE mRNA expression levels in different cell lines. (C) The expression levels of YWHAE in colorectal samples from the TCGA database. *, P<0.05. CRC, colorectal cancer; PCT, para-carcinoma tissue; TCGA, The Cancer Genome Atlas; YWHAE, 14-3-3ε.

in SW620 cells. Therefore, the next step in this study was to explore the effect of altered YWHAE expression on tumorigenesis and development in SW620 cells.

Then, the information was downloaded from TCGA database for 51 normal tissue samples (including 41 colon samples and 10 rectal samples) and 644 tumor tissue samples (including 478 colon samples and 166 rectal samples). The results showed that the expression level of YWHAE in CRC tumor tissues was significantly higher than that in para-carcinoma tissues (*Figure 1C*).

Efficiency of YWHAE silencing and overexpression

In order to investigate the effect of altered *YWHAE* gene expression on the function of CRC cell line SW620, plko.1-sh-YWHAE and psin-YWHAE + Flag plasmids were constructed and infected SW620 cells through lentivirus. Western blot and RT-qPCR results showed that the expression levels of protein and mRNA in YWHAE silenced cells were significantly decreased (*Figure 2A-2C* and Figure S1B), while the expression levels of protein and mRNA in YWHAE overexpressed cells were significantly increased (*Figure 2D-2F* and Figure S1C). The results showed that $SW620^{sh\text{-}YWHAE}$ (by plko.1-sh-YWHAE plasmid) and $SW620^{YWHAE\ vector}$ (by psin-YWHAE + Flag plasmid) were successfully constructed.

Effect of altered YWHAE expression on the proliferation, migration and apoptosis of SW620 cells

To explore the effect of altered YWHAE expression on the proliferation of SW620 cells, CCK-8 and EdU were used to detect cell proliferation. Compared with the control group, the results of CCK-8 showed that silenced group cells grew significantly slower, while cells in the overexpressed group grew faster all the time, and there was a significant difference between the two groups (*Figure 3A*), indicating that silenced YWHAE inhibited the growth of cells. The EdU results more obviously illustrated this point. Compared with the control group, the cells in the silent group grew significantly slower, while the cells in the overexpressed group grew faster (*Figure 3B,3C*). In conclusion, YWHAE promoted the proliferation of SW620 cells.

Next, this study investigated the effect of altered YWHAE expression on the migration and apoptosis of SW620 cells. Compared with the control group, the scratch healing was slower in the expression silence group, and YWHAE could improve the migration ability of SW620 cells (*Figure 3D,3E*). Transwell results showed that YWHAE promoted cell migration and metastasis (*Figure 3F,G*). Flow cytometry results showed that apoptosis of the silenced group was significantly increased, while apoptosis was decreased in the overexpressed group (*Figure 3H,3I*). In summary, in SW620 cells, YWHAE promoted the proliferation and migration of CRC cells, inhibited apoptosis, and played the function of proto-oncogene.

YWHAE affects the proliferation, migration and apoptosis of SW620 cells by regulating the Wnt/β-catenin signaling pathway

To explore the molecular mechanism of YWHAE's influence on the proliferation, migration and apoptosis of SW620 cells, the expression level of genes related to the β-catenin signaling pathway, which is the central mechanism of CRC occurrence, was detected in this study. Compared with the control group, the expression level of β-catenin

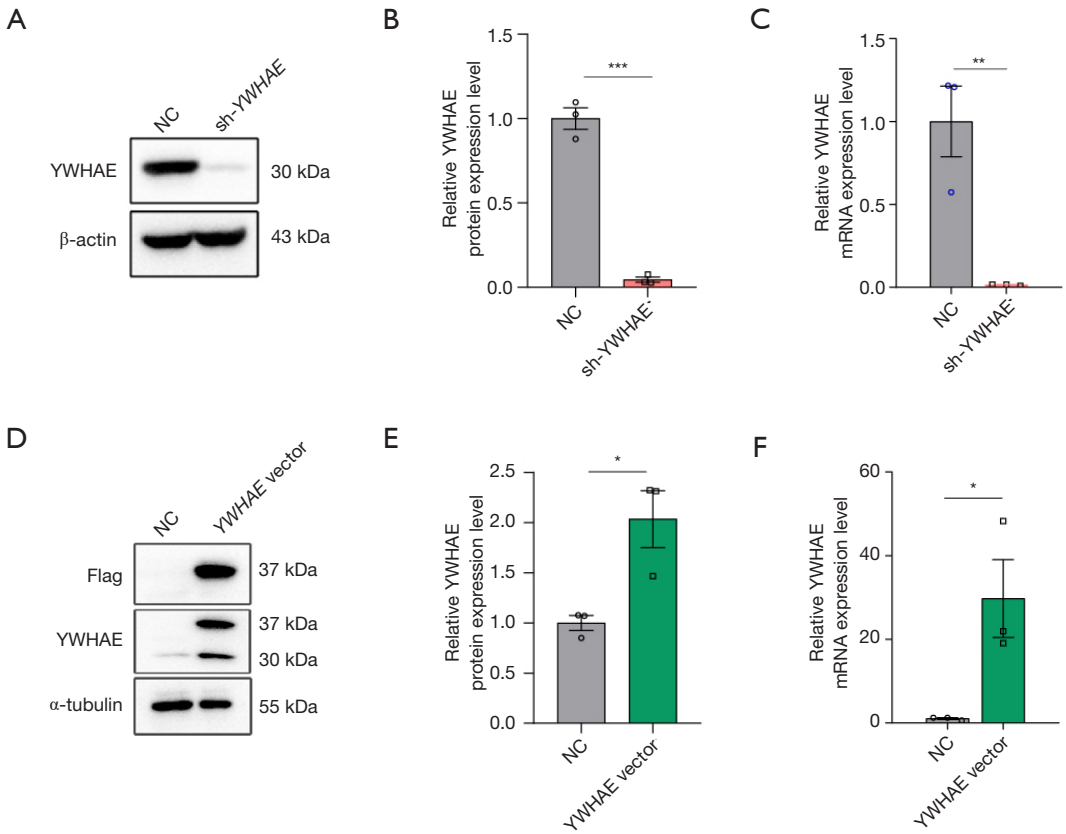

Figure 2 Efficiency of *YWHAE* silencing and overexpression. (A,B) The protein expression levels of YWHAE in silenced cells (A) and the quantitative analysis (B). (C) The mRNA expression levels of YWHAE in silenced cells. (D,E) The protein expression levels of YWHAE in overexpressed cells (D) and the quantitative analysis (E). (F) The mRNA expression levels of YWHAE in overexpressed cells. *, P<0.05; **, P<0.01; ***, P<0.001. NC: negative control; sh-YWHAE: silenced YWHAE expression; YWHAE vector: YWHAE overexpression. YWHAE, 14-3-3ε.

protein in expression-silenced cells was significantly decreased, while the expression level of β-catenin protein in overexpressed cells was significantly increased (*Figure 4A-4C* and Figure S1D). In addition, the mRNA expression levels of E-cadherin, cyclin D, c-myc and p-120 catenin in expression-silenced cells were significantly decreased (*Figure 4D-4G*), while the mRNA expression levels of vimentin were not significantly decreased (*Figure 4H*). In the overexpressed cells, only the mRNA of p-120 catenin increased, and the mRNA expression of other genes showed no significant difference, which might be due to the high level of YWHAE expression in the background of SW620 cells (*Figure 4C-4H*).

YWHAE affects the proliferation, migration and apoptosis of SW620 cells by regulating EVs secretion

Next, we explored whether *YWHAE* affected the proliferation, migration and apoptosis of CRC by influencing EVs secretion. The results of NTA showed that the silencing and overexpression of *YWHAE* had no effect on the particle size distribution of EVs, and the particle size basically showed a normal distribution (*Figure 5A-5C*). By analyzing the number of EVs particles secreted by the same number of cells, the number of EVs secreted by expression-silenced cells was significantly reduced, while the number of EVs secreted by overexpressed cells was significantly

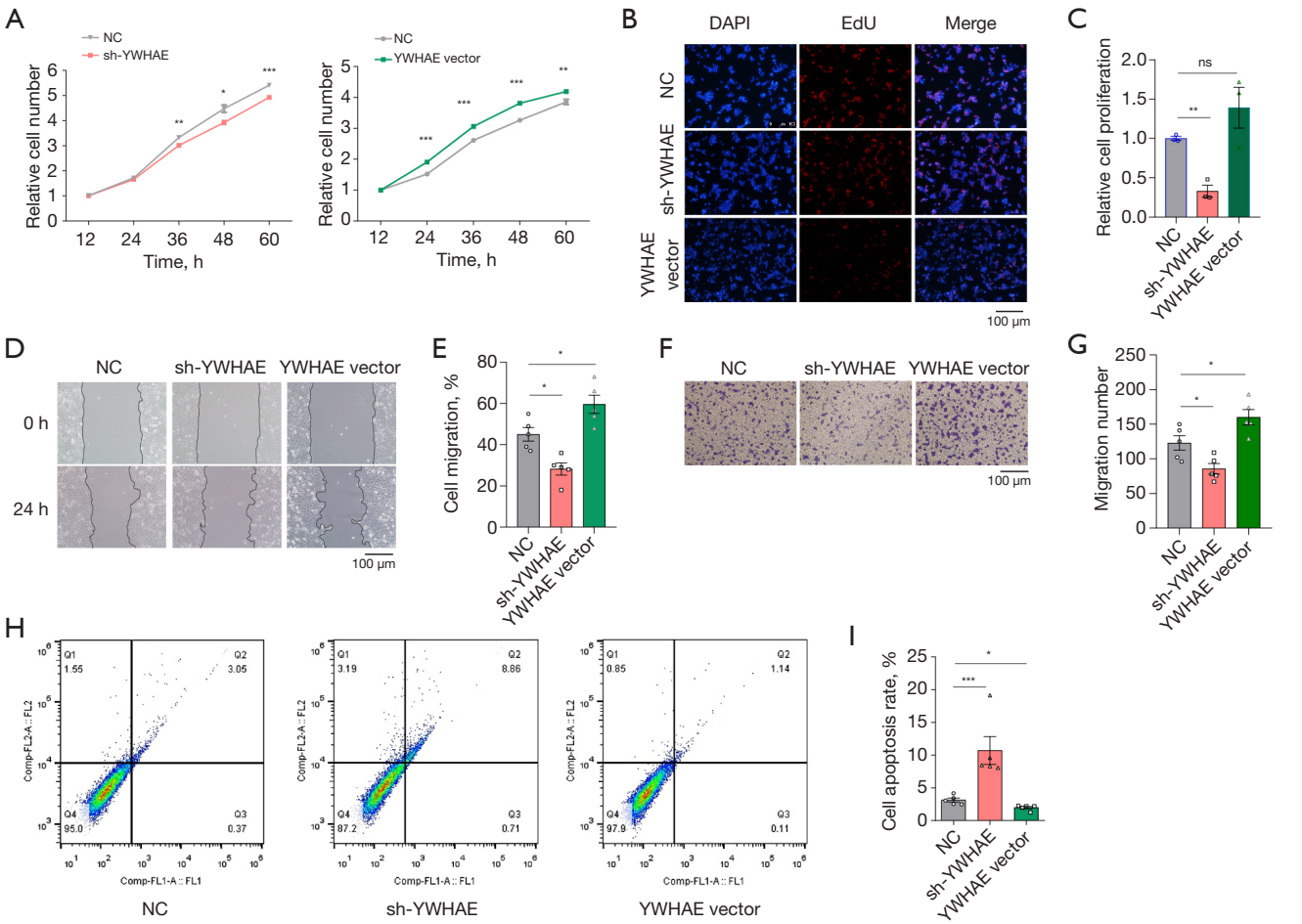

Figure 3 Effect of *YWHAE* silencing or overexpression on the proliferation, migration and apoptosis of SW620 cells. (A) The effect on tumor cell proliferation was assessed by CCK-8 assay. (B) The effect on cell proliferation was analyzed by EdU staining (scale =100 μm). (C) Quantitative analysis of EdU staining results. (D) The effect on cell migration was evaluated by scratch assay (scale =100 μm). (E) Quantitative analysis of scratch assay results. (F) The effect on cell migration was assessed by Transwell assay (scale =100 μm, cells were dyed with 0.1% crystal violet). (G) Quantitative analysis of Transwell assay results. (H) The effect on apoptosis was assessed by flow cytometry. (I) Quantitative analysis of flow cytometry results. ns, no significant difference; *, P<0.05; **, P<0.01; ***, P<0.001. NC: negative control; sh-YWHAE: silenced YWHAE expression; YWHAE vector: YWHAE overexpression. CCK-8, Cell Counting Kit-8; EdU, 5-ethynyl-2'-deoxyuridine; YWHAE, 14-3-3ε.

increased (*Figure 5D*). Western blot results showed that the EVs membrane proteins secreted by the same number of cells (CD63, CD9) were not affected in YWHAE silenced and overexpressed cells, while the proteins involved in EVs formation (Alix, Tsg101) were not significantly changed in *YWHAE* silenced cells. It was slightly increased in YWHAE overexpressing cells. However, β-catenin secreted protein was significantly decreased in EVs of YWHAE silenced cells and significantly increased in EVs of YWHAE overexpressed cells (*Figure 5E-5G* and Figure S1E).

YWHAE affects the secretion of EVs from CRC to tumor microenvironment (TME) cells

Next, this study selected human bronchial epithelial cell line HBE as the recipient cell to further verify *in vitro* whether altered YWHAE expression in CRC cells affected EVs secretion from the cells into TME (*Figure 6A*). The CCK-8 results showed that compared with the control group, EVs from silenced cells inhibited the viability of HBE cells, and EVs from overexpressed cells promoted the viability of HBE cells (*Figure 6B*). EdU results showed that compared

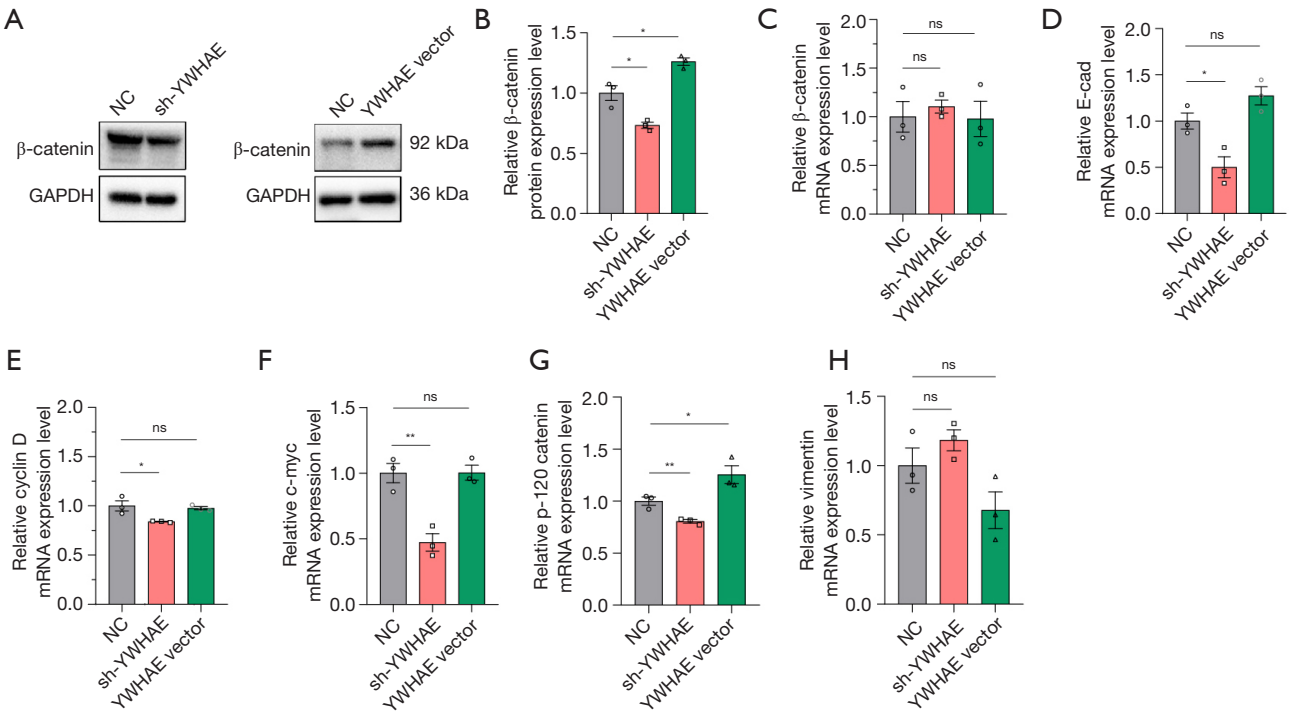

Figure 4 Effect of *YWHAE* silencing or overexpression on β-catenin signaling pathway in SW620 cells. (A,B) Expression level (A) and quantitative analysis (B) of β-catenin protein in silenced and overexpressed cells. (C) The expression level of β-catenin mRNA in silenced and overexpressed cells. (D) The expression level of E-cadherin mRNA in silenced and overexpressed cells. (E) The expression level of cyclin D mRNA in silenced and overexpressed cells. (F) The expression level of c-myc mRNA in silenced and overexpressed cells. (G) The expression level of p-120 catenin mRNA in silenced and overexpressed cells. (H) The expression levels of vimentin mRNA in silenced and overexpressed cells. ns, no significant difference; *, P<0.05; ** P<0.01. NC: negative control; sh-YWHAE: silenced YWHAE expression; YWHAE vector: YWHAE overexpression. YWHAE, 14-3-3ε.

with the control group, EVs from silenced cells inhibited proliferation of HBE cells, and EVs from overexpressed cells promoted proliferation of HBE cells (*Figure 6C,6D*). Transwell results showed that compared with the control group, EVs from silenced cells inhibited the migration of HBE cells, and EVs from overexpressed cells promoted the migration of HBE cells (*Figure 6E,6F*).

Discussion

CRC is a common gastrointestinal tumor, and the incidence of CRC is getting younger year by year (20). Furthermore, a quarter of CRC patients have metastases at diagnosis, and approximately 35% to 45% of CRC patients with metastases relapse within 5 years of surgery (21). Therefore, exploring the molecular mechanism of the occurrence and development of CRC is the key to improve the survival rate of patients.

In recent years, YWHAE protein has been found to be abnormally expressed in various tumors, including CRC. YWHAE has a variety of bioactive functions, and is involved in various cell physiological activities such as apoptosis, growth and metabolism regulation, cell cycle and phosphorylation-dependent protein transport process. It may participate in the occurrence and development of tumors by activating multiple signaling pathways such as NF-κB, Ras-Raf and PI3K/AKT/mTOR. In addition to mediating tumor development as a competing endogenous RNA, YWHAE can also influence gene expression through chromosomal variations. For example, *YWHAE-FAM22* fusion gene induced by t(10; 17)(q22; p13) may be associated with the development of endometrial stromal sarcoma (ESS) (22). The *YWHAE-FAM22* fusion gene transcript is specific and can be used as an effective auxiliary diagnostic method to confirm the diagnosis of ESS. Meanwhile, fusion transcripts of *YWHAE* and *NUTM2*

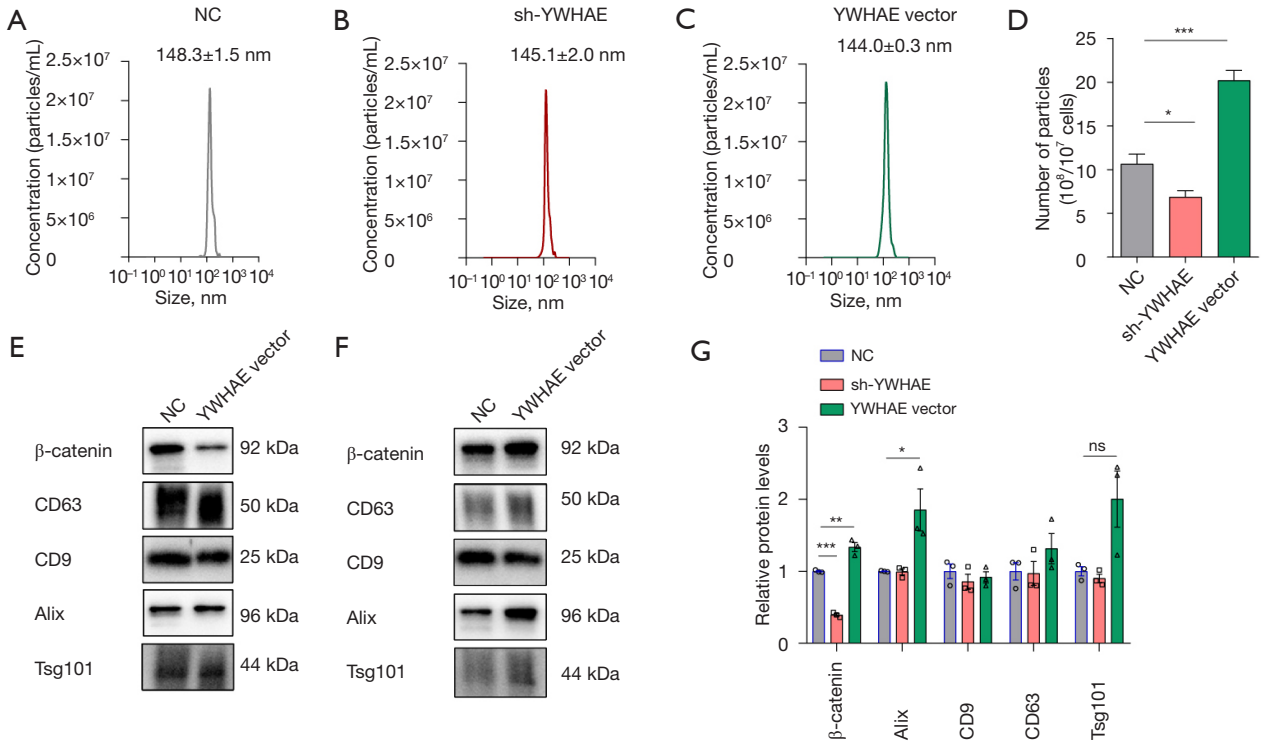

Figure 5 Effect of *YWHAE* silencing or overexpression on EVs secretion in SW620 cells. (A-D) The diameter distribution (A-C) and particle number (D) of EVs from an equal number of control cells, *YWHAE*-silenced cells and *YWHAE*-overexpressed cells were analyzed by NTA. (E-G) The western blot (E,F) and quantitative analysis (G) of β-catenin, CD63, CD9, Alix, and Tsg101 expression in *YWHAE*-silenced and -overexpressed cells. ns, no significant difference; *, P<0.05; ** P<0.01; ***, P<0.001. NC: negative control; sh-YWHAE: silenced YWHAE expression; YWHAE vector: YWHAE overexpression. EVs, extracellular vesicles; NTA, nanoparticle tracking analysis; YWHAE, 14-3-3ε.

lead to the development of ESS (23). The polymorphism of *YWHAE* gene and the variation in the 17p13.3 region are also associated with the occurrence of mental illness. In general, the gene fusion is closely related to the prognosis of tumor diseases (5,24). Liu *et al.* reported that pan-cancer analysis revealed elevated expression of YWHAE in several cancer types compared to normal tissues. They utilized the gene set cancer analysis (GSCA) database to assess the methylation, single nucleotide variations (SNVs), and copy number variations (CNVs) associated with *YWHAE* (25). Somatic mutation analysis illustrated that missense mutations and multi-hit variants are the most common types of mutations, with a significant pro-portion of SNVs being C>T mutations. Results showed that heterozygous deletion and homozygous amplification are the most prevalent types of CNVs. The correlation analysis between CNVs and *YWHAE* gene expression revealed a positive correlation in 29 out of 33 cancers (25). Moreover, they also found a positive correlation between *YWHAE* gene expression and the tumor mutation burden. Among the 557 genes that showed a positive correlation with YWHAE, 31 genes that associated with prognosis were identified. Based on the evaluation index, C-index, Enet [0.2] was selected as the final algorithm. By applying 10 machine learning algorithms and 101 integration combinations, they developed a prognostic model for *YWHAE*-related genes. Subsequently, 14 genes (such as *ERCC6L*, *TAF5*, etc.) for constructing the prognostic model were identified by Enet [0.2] (25).

To further verify the relationship between *YWHAE* gene and the occurrence and development of CRC, this study constructed YWHAE expression-silenced cells and overexpression cells to verify the functional changes from the malignant biological behavior of tumor cells. Then, the YWHAE expression profile and clinicopathological characteristics were downloaded from the TCGA database

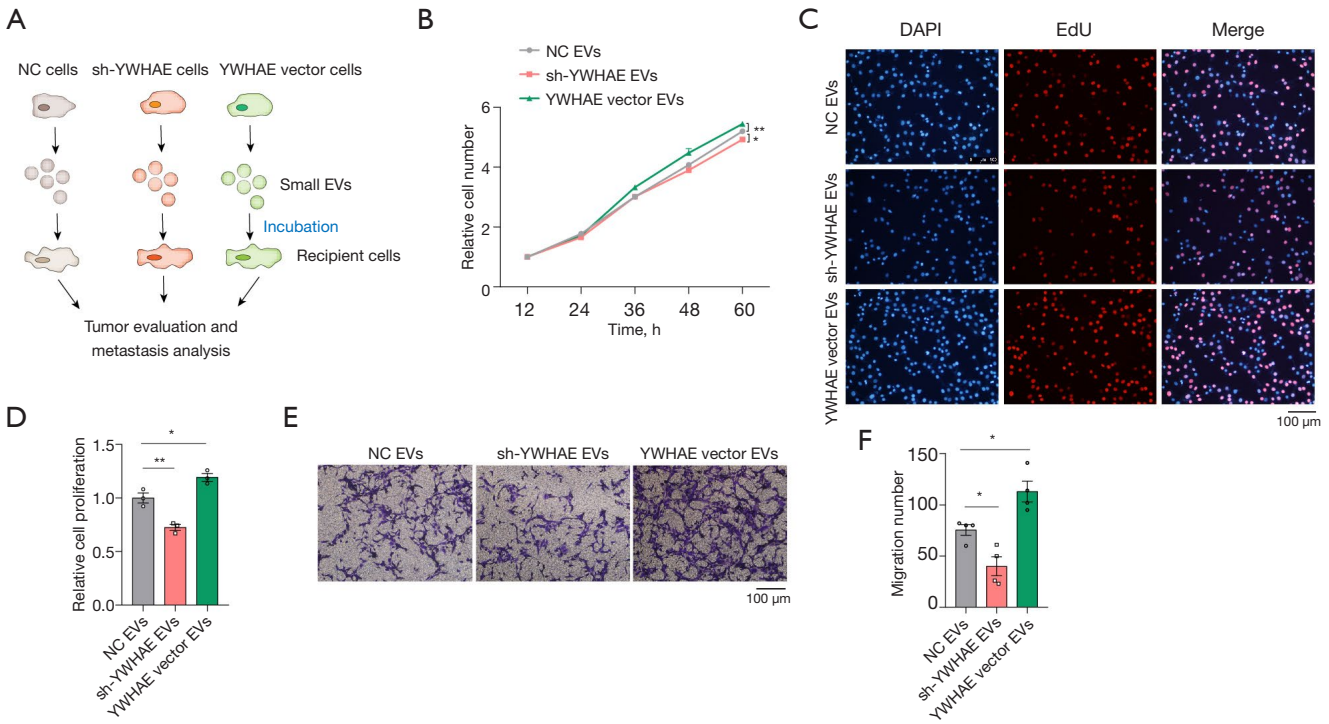

Figure 6 Effects of EVs secreted by CRC cells of different YWHAE expression levels on recipient HBE cells. (A) Experimental design flow chart: EVs were isolated from donor control cells and culture medium, and then incubated with recipient HBE cells. The proliferation and metastasis ability of HBE cells were analyzed. (B) The effect of secreted EVs on tumor cell proliferation was detected by CCK-8. (C) The effect of EVs on cell proliferation was detected by EdU staining (scale =100 μm). (D) Quantitative analysis of EdU staining results. (E) The effect of EVs on cell migration was detected by Transwell assay (scale =100 μm, cells were dyed with 0.1% crystal violet). (F) Quantitative analysis of Transwell assay results. *, P<0.05; ** P<0.01. NC: negative control; sh-YWHAE: silenced YWHAE expression; YWHAE vector: YWHAE overexpression. CCK-8, Cell Counting Kit-8; CRC, colorectal cancer; EdU, 5-ethynyl-2'-deoxyuridine; EVs, extracellular vesicles; YWHAE, 14-3-3ε.

for analysis, and it was found that the expression of YWHAE in cancer tissues was significantly higher than that in normal tissues. However, it was reported that further analysis of clinicopathological characteristics showed that YWHAE expression was not correlated with gender, age, pathological stage, depth of invasion, lymph node metastasis, and distant metastasis of patients, and neither high-expression group nor low-expression group was correlated with survival status of patients (26). These provide guidance for the subsequent diagnosis and prognosis of CRC.

The Wnt signaling pathway is a relatively conserved signaling pathway in evolution and plays a key role in embryonic development, cell proliferation, differentiation and orientation. The Wnt signaling pathways mainly include Wnt/β-catenin, Wnt/Ca^{2+} and Wnt/planar cell polarity signaling pathways, among which Wnt/β-catenin signaling pathway is the most critical and classic (27). Abnormal activation of the Wnt/β-catenin signaling pathway has been implicated in growth-related diseases and cancers and has important biological significance in tumorigenesis and prognosis, particularly as a key driver of CRC development and progression (28,29). It plays an important role in chemotherapy resistance, cell proliferation and apoptosis. Several inhibitors of Wnt/β-catenin signaling pathway have been developed for the treatment of CRC and have potential value as therapeutic targets for CRC (30). This study found that after YWHAE expression was silenced, CRC cells' proliferation and migration activity were weakened, and the apoptosis rate was increased, indicating that YWHAE functioned as a proto-oncogene in CRC tissues and cells and promoted the development of tumors. The mechanism might be that the inhibition of YWHAE is related to the decreased expression of related genes (β-catenin, E-cadherin, cyclin D, c-myc, p-120

catenin, and vimentin) in the Wnt/β-catenin signaling pathway. However, the mRNA expression of related genes in the Wnt/β-catenin signaling pathway was not significantly different in overexpressed cells, which may be due to the high level of YWHAE expression in the background of SW620 cells.

With the deepening of research related to EVs, the pathogenic mechanisms of many diseases are revealed. Through follow-up experiments and in-depth studies on the biological effects and signaling pathways of EVs, EVs is expected to become a more valuable new therapeutic means in the prevention, diagnosis, treatment, and prognosis of various diseases (31). The properties of EVs are suitable for the delivery of a variety of therapeutic drugs, including proteins, nucleic acid drugs, targeted drugs, and gene-editing drugs (32,33). In this study, the ability of expression-silenced cells to secrete EVs was weakened, and the expression level of β-catenin in EVs was significantly increased, which inhibited the proliferation activity of TME cells. The ability of overexpressing cells to secrete EVs was enhanced, and the expression level of β-catenin encapsulated by EVs was significantly increased, which enhanced the proliferation activity of TME cells. In future studies, specific modifications of EVs, delivery of therapeutic protein drugs, nucleic acid drugs, etc., can directly achieve specific targeted therapy, inhibit the release of specific EVs, and thus inhibit the occurrence and development of diseases.

In summary, YWHAE is highly expressed in CRC cells and tissues, and silencing YWHAE expression can inhibit the proliferation and migration of CRC and promote apoptosis, which may be related to reducing the expression of related genes in Wnt/β-catenin signaling pathway and inhibiting the secretion of EVs in tumor cells. This study confirmed the role of YWHAE in the occurrence and development of CRC.

Conclusions

In conclusions, our study demonstrates that silencing YWHAE expression can inhibit the proliferation and migration of CRC cells and promote cell apoptosis. Mechanistically, it is related to the inhibition of Wnt/β-catenin signaling and EVs secretion.

Acknowledgments

None.

Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://amegroups.com/article/view/10.21037/tcr-24-1910/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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