Article

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Knocking Down *WARS1* in Colorectal Cancer: Implications for Apoptosis and Cell Cycle Arrest via the p53 Signaling Pathway

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Background: Preventing the progression and recurrence of colorectal cancer (CRC) remains a clinical challenge due to its heterogeneity and drug resistance. This underscores the need to discover new targets and elucidate their cancer-promoting mechanisms. This study analyzed the cancer-promoting mechanisms of tryptophanyl-tRNA synthetase 1 (*WARS1*) in CRC.
Methods: Clinical data and RNA expression profiles of CRC patients in public databases were analyzed using bioinformatics to determine the expression of *WARS1*. A *WARS1* knockdown assay was conducted with HCT116 and RKO cell lines to systematically assess the effects of *WARS1* on CRC cell proliferation, migration, cell cycle, and apoptosis. These assessments employed reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Western blotting, wound healing and transwell assays, flow cytometry, and xenograft tumor assays. Additionally, RNA sequencing and gene enrichment-based analysis were performed following *WARS1* knockdown to detect gene expression changes and related pathways.
Results: *WARS1* was overexpressed in CRC tissues ($p < 0.05$). Downregulation of *WARS1* inhibited the growth and migration of RKO and HCT116 cell lines ($p < 0.05$). This inhibitory effect on tumor growth was also observed in xenografts in nude mice after *WARS1* knockdown ($p < 0.01$). Flow cytometry revealed an increase in apoptosis and cell cycle arrest following *WARS1* knockdown ($p < 0.05$). Transcriptome sequencing analysis showed that reduced expression of *WARS1* activated the p53 signaling pathway and apoptosis while suppressing DNA replication and the cell cycle. The p53 transcriptional inhibitor pifithrin-α partially prevented the activation of caspase 3 and reduced the levels of c-poly-ADP-ribose polymerases 1 (*PARP1*) and cyclin-dependent kinase inhibitor 1A (*CDKN1A*).
Conclusion: *WARS1* was highly expressed in CRC, and its low expression was identified as a risk factor for CRC progression and recurrence. The current findings provide a theoretical basis for the development of therapeutic agents targeting *WARS1* and elucidate its mechanism in CRC progression.

Keywords: colorectal cancer; *WARS1*; p53 pathway; apoptosis; cell cycle

Introduction

Colorectal cancer (CRC) is the third most frequent malignancy and the second leading cause of cancer mortality [1,2]. In China, the incidence and death rate of CRC are slightly higher than the global average [3]. Current clinical treatment options for CRC include surgery, chemoradiotherapy, targeted therapy [4], and immunotherapy [5]. However, treatment efficacy and CRC prognosis are influenced by various factors such as heterogeneity [6], drug resistance [7], and metastasis [8]. Screening for new targets and studying their molecular mechanisms in CRC progression can help elucidate CRC pathogenesis and improve clinical treatment and diagnosis.

Tryptophanyl-tRNA synthetase (WARS) is a crucial enzyme involved in catalyzing the ligation of tryptophan to its cognate tRNA during translation. WARS can expand its functional repertoire through mechanisms such as alternative splicing, proteolytic cleavage, and even extracellular activity [9]. There are two isoforms of WARS: the cytoplasmic isoform (*WARS1*) and the mitochondrial isoform (*WARS2*). Evidence suggests that interferon gamma (*IFN-*γ) regulates *WARS1* expression to mediate innate inflammatory responses [10], inhibit angiogenesis, and control vascular permeability [11], potentially contributing to early sepsis detection [12]. Additionally, *WARS1* mediates resveratrol to promote $CD8^+$ T-cell proliferation [13], increases chemotherapy sensitivity in hormone receptor-positive breast cancer [14], promotes the progression of uveal melanoma [15], and influences the metastatic abil-

ity of pancreatic cancer cells [16]. However, the impacts of *WARS1* on CRC cell growth and migration, as well as the fundamental mechanisms underlying these processes, have not been reported.

By accessing public datasets from various sources, this study investigated changes in *WARS1* expression in CRC and its potential associations with recurrence and prognosis. The regulatory impact of *WARS1* on the proliferation and migration of CRC cell lines was assessed. Furthermore, molecular analyses, including RNA sequencing and diverse molecular techniques, were conducted to explore potential underlying mechanisms. The present findings could inspire the future development of therapeutic agents targeting *WARS1* in CRC.

Materials and Methods

Bioinformatics Analysis

Clinical data of CRC patients and their corresponding RNA expression profiles were sourced from The Cancer Genome Atlas (TCGA (https://portal.gdc.cancer.gov/)) via the University of California Santa Cruz (UCSC) Xena platform. Additional datasets, including GSE9348, GSE20916, GSE24514, GSE41258, GSE8671, GSE110223, and GSE39582, were retrieved from the Gene Expression Omnibus (GEO (https://www.ncbi.nlm.nih.gov/gds/)) database. Notably, only samples with complete clinical information and RNA sequencing data were included, while duplicates with poor sequencing quality were excluded.

The fragments per kilobase million (FPKM) expression values were converted to transcripts per kilobase million (TPMs). The expression levels of *WARS1* in various cancer tissues and their corresponding normal tissues were analyzed and visualized using the "ggplot2" package in R software (v. 4.2.1, NIHR Great Ormond Street Hospital Biomedical Research Centre, London, UK).

The protein levels of *WARS1* were evaluated using the UALCAN [17] and Human Protein Atlas database [18]. To assess the expression of *WARS1* as a prognostic indicator for CRC, the "survfit" function in the R package "survival" (v. 3.6-4, University of Auckland, Auckland, New Zealand) was employed. For further analysis, Gene Set Enrichment Analysis (GSEA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed using the "clusterProfiler" package (v. 0.4.6, Baylor College of Medicine, Houston, TX, USA) [19,20].

Cell Lines and Culture Conditions

The cells were authenticated using the current Short Tandem Repeat (STR) typing technique based on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Leibniz Institute, Braunschweig, Germany). Additionally, mycoplasma detection results yielded negative findings. The HCT116 (BNCC287750, BeNa Culture Collection, Xinyang, China) and RKO (BNCC100173, BeNa Culture Collection, Xinyang, China) cell lines were chosen as representative human CRC models due to their distinct genetic backgrounds. This selection allowed for effective exploration of the role of *WARS1* in CRC progression and identification of potential therapeutic targets.

In this study, the human CRC cell lines HCT116 and RKO (purchased from BeNa Culture Collection, Xinyang, China) were cultured at 37 °C in a 5% CO_2 environment in high-glucose DMEM supplemented with L-glutamine (Tc-G8320, HyClone, Logan, UT, USA), 10% fetal bovine serum (FBS) (A5670701, Gibco, Thermo Fisher Scientific, Inc., Carlsbad, CA, USA), and 1% penicillin-streptomycin solution (10 µL/mL) (15140122, Gibco, Thermo Fisher Scientific, Inc.).

Generation of WARS1 Knockdown Lines

For the transient interference experiment, a mixture comprising 4 µL of Lipo8000 (C0533, Beyotime, Shanghai, China) and 80 pmol of shRNA (GenePharma, Suzhou, China) (detailed in **Supplementary material 1**) was gently combined and subsequently added to 5.0×10^5 cells. Following a 72-hour incubation period, the cells were lysed for further analysis. Subsequently, to investigate the function of *WARS1* in CRC cells, short hairpin RNA (shRNA)-mediated gene silencing was employed to knock down *WARS1* in HCT116 and RKO cell lines. This method enabled the persistent suppression of *WARS1* expression in cells, facilitating the observation of its effects on the biological properties of cancer cells. All cells were transfected with a lentiviral vector LV10N-Puro-WARS1-Homo-1240 (GenePharma, Suzhou, China) at a multiplicity of infection (MOI) of 10. The knockdown cells were selected through exposure to 1 µg/mL puromycin (ST551, Beyotime, Shanghai, China) for 3 days and prepared for further experiments.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Changes in the expression of *WARS1* at the mRNA level were detected using RT-qPCR. Total RNA was extracted using Trizol (R0016, Beyotime, Shanghai, China), followed by RT-qPCR using the BeyoFast SYBR Green One-Step qRT-PCR Kit (D7260, Beyotime, Shanghai, China) with a 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). $2^{-\Delta\Delta Ct}$ was used for calculating gene expression. The experiment was conducted strictly following the protocols provided by the manufacturer.

The primer sequences (**Supplementary material 2**) were synthesized by GENEWIZ (South Plainfield, NJ, USA).

Western Blotting

Cell lysis was performed using RIPA lysis buffer (P0013B, Beyotime, Shanghai, China), and the total protein was subsequently separated via SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (IPVH00010, Millipore, Billerica, MA, USA). Following a 2-hour blocking step with 5% nonfat milk, the membranes were incubated with the following primary antibodies: WARS1 (Cat No. 67952-1-Ig), phospho-p53 (Ser15) (Cat No. 28961-1-AP), tumor protein p53 inducible protein 3 (TP53I3)/p53-inducible gene 3 (PIG3) (Cat No. 14828-1-AP), cell division cycle 6 (CDC6) (Cat No. 66021-1-Ig), cell division cycle 45 (CDC45) (Cat No. 15678-1-AP), poly-ADP-ribose polymerases 1 (PARP1) (Cat No. 66520-1-Ig), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat No. 60004-1-Ig) (Proteintech, Rosemont, IL, USA); and p53 (Cat No. AF0255), BCL2 binding component 3 (BBC3) (alias PUMA) (Cat No. AF0270), cyclin-dependent kinase inhibitor 1A (CDKN1A) (Cat No. AF5252), Minichromosome Maintenance Complex Component 3 (MCM3) (Cat No. AG2608), Minichromosome Maintenance Complex Component 7 (MCM7) (Cat No. AF7434), and c-Caspase-3 (CASP3) (Cat No. AF1150) (Beyotime, Shanghai, China).

The PVDF membranes were then incubated for 1 hour with secondary antibodies, including horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:2000 dilution, Beyotime, Shanghai, China, Cat# A0208) and HRP-labeled goat anti-mouse IgG (1:2000 dilution, Beyotime, Shanghai, China, Cat# A0216). After color development using the ECL Kit (P0018M, Beyotime, Shanghai, China), the protein bands were visualized and captured using a gel imaging system (Bio-Rad, ChemiDoc MP, Boston, MA, USA). The gray values of the bands were analyzed for their intensity using ImageJ software (Version 1.8.0, National Institutes of Health, Madison, WI, USA).

Wound-Healing Assay

The wound-healing assay is a widely utilized method to evaluate cell migration ability under *in vitro* conditions. This assay involves observing the rate at which cells migrate to fill a created "wound" area.

Cells were cultured in 6-well plates until 80% confluent. Subsequently, a wound was carefully formed using a 200-µL pipette tip, and any cellular debris was gently removed using a culture medium. After incubation in a medium containing 1% serum for 24 hours, the reduced wound area was photographed using an Olympus camera (BX53M, Olympus, Tokyo, Japan) and measured using ImageJ software developed by the National Institute of Health.

Transwell Assay

The transwell assay was conducted to further validate the effect of *WARS1* on the migration ability of CRC cell lines. Cells at a density of 2×10^4 were suspended in 200 µL of serum-free medium and then transferred into the upper chamber of a transwell plate (353097, Corning, Corning, NY, USA), while 600 µL of culture medium containing 10% FBS was added to the lower chamber.

After 36 hours of incubation, the cells in the upper chamber were carefully cleaned using a cotton swab, and the remaining cells were fixed and stained. The stained cells were then observed and recorded using an inverted microscope (CKX53, Olympus, Tokyo, Japan).

Cell Growth Assay

Cells were inoculated into separate 6-well plates at a density of 4.0×10^4 cells/well. After 24, 48 and 72 hours of incubation, the cells were quantified to analyze the effects of *WARS1* on CRC cell growth. Additionally, the DepMap portal (https://depmap.org/portal/) [21] was utilized to further investigate the impact of *WARS1* on CRC cell growth.

Flow Cytometry Assay

All cells were inoculated into Petri dishes and maintained at 37 °C with 5% CO_2 to ensure optimal conditions for the experiments. After trypsinization, the cells were stained with Annexin V FITC-PI and analyzed using flow cytometry with the Annexin V FITC/PI Apoptosis Detection Kit from BD Biosciences (556570, Franklin Lake, NJ, USA), following the provided guidelines.

Cellular analysis was conducted using a CyFlow Cube 8 flow cytometer (Sysmex, Franklin Lake, NJ, USA), followed by data interpretation using the FCS Express V3 software (De Novo Software, Kitchener, Ontario, Canada) and FlowJo (version 10.8.1) software (TreeStar, Woodburn, OR, USA).

RNA Sequencing

A total of 5×10^6 cells from each sample were collected and sent to the NovelBio Cloud Analysis platform (Shanghai, China) for transcriptome sequencing, with three replicates for each group.

Animal Experiments

To emulate the *in vivo* tumor environment more accurately, we employed a nude mouse model (age 5–8 weeks, 16–18 g) to validate the clinical relevance of the *in vitro* results. Twelve male BALB/c nude mice aged between 8 and 12 weeks were obtained from Beijing Vital River Laboratory Animal Technology (Beijing, China). These mice were randomly divided into two groups: the experimental group and the control group, with six animals in each group.

Using a sterile syringe, 1.0×10^7 shWARS1-RKO cells and control cells were injected subcutaneously into the left axilla of the experimental and control mice, respectively. The injection procedure was conducted under sterile conditions at a fixed site and at a constant speed.

At the conclusion of the experiment (day 28), the mice underwent isoflurane inhalation anesthesia at a concentra-

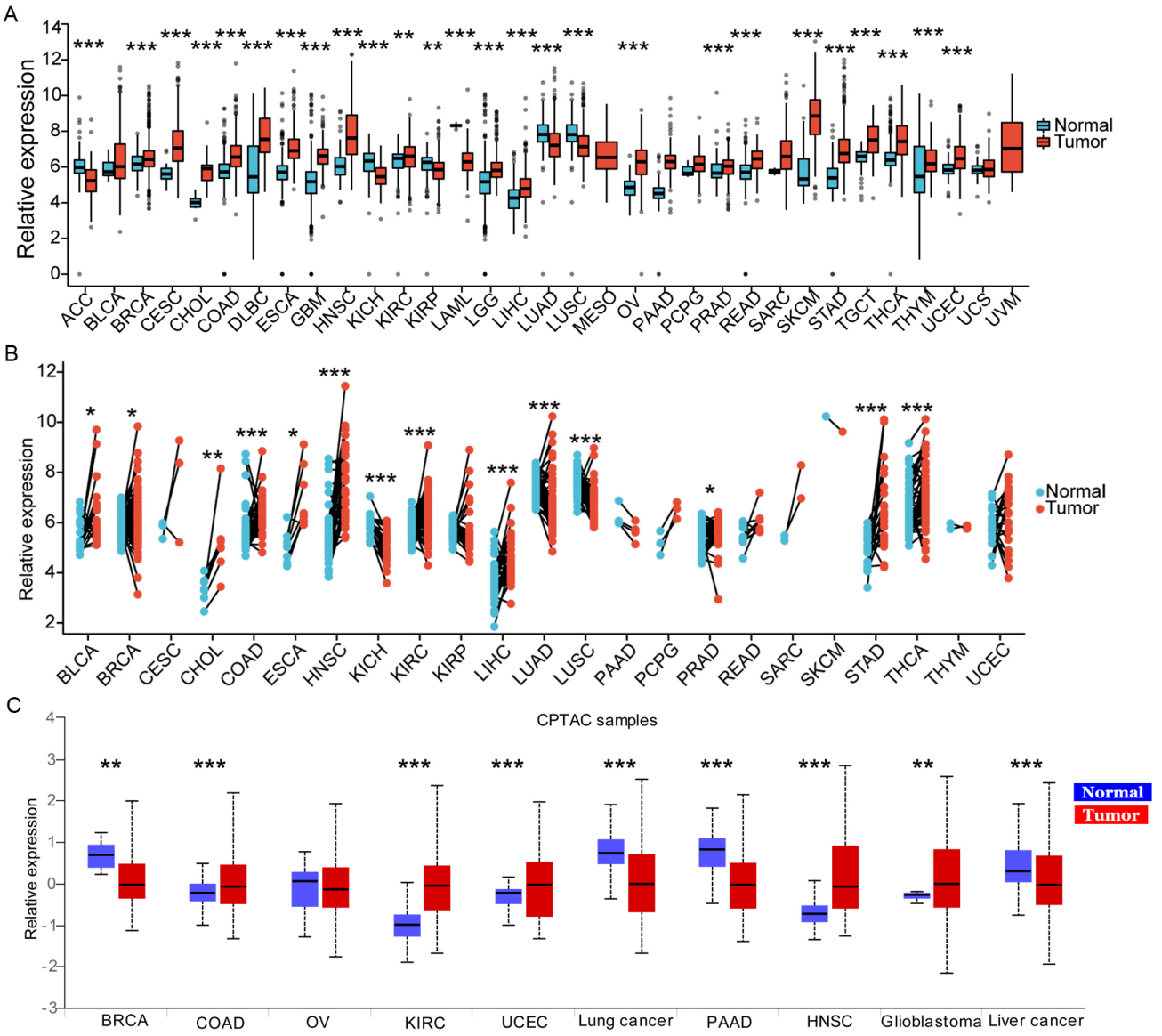

Fig. 1. Pan-cancer analysis of tryptophanyl-tRNA synthetase 1 (*WARS1*) expression. (A) The mRNA level of *WARS1* in unpaired samples from The Cancer Genome Atlas (TCGA). (B) The mRNA level of *WARS1* in paired samples from TCGA. (C) The protein level of *WARS1* in pan-cancer samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

tion of 4%–5% for approximately 3 minutes. Subsequently, they were placed in an airtight transparent container and euthanized with an overdose of CO_2 at a flow rate of 30%–70% volume/minute to ensure gradual loss of consciousness without pain. Finally, the tumors were excised from the left axilla of the mice using sterilized surgical tools, weighed, and recorded.

Statistical Analyses

Statistical analyses were performed using R software (v. 4.2.1, NIHR Great Ormond Street Hospital Biomedical Research Centre, London, UK) and GraphPad Prism (v. 9.5.1) (GraphPad Software Inc., San Diego, CA, USA). Differences between unpaired samples were calculated using the unpaired *t*-test, while paired samples were analyzed using the paired *t*-test. The chi-square test was employed to analyze differences in *WARS1* expression across different stages. Survival curves were generated through Kaplan-Meier analysis and subsequently evaluated using the log-rank test. Statistical significance was defined at $p < 0.05$.

Results

Colorectal and Other Cancers Highly-Expressed WARS1

Analysis of unpaired pan-cancer samples in TCGA revealed significant overexpression of the *WARS1* gene in 21 cancers, including colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) ($p < 0.05$; Fig. 1A). Similarly, paired sample analysis demonstrated significant up-

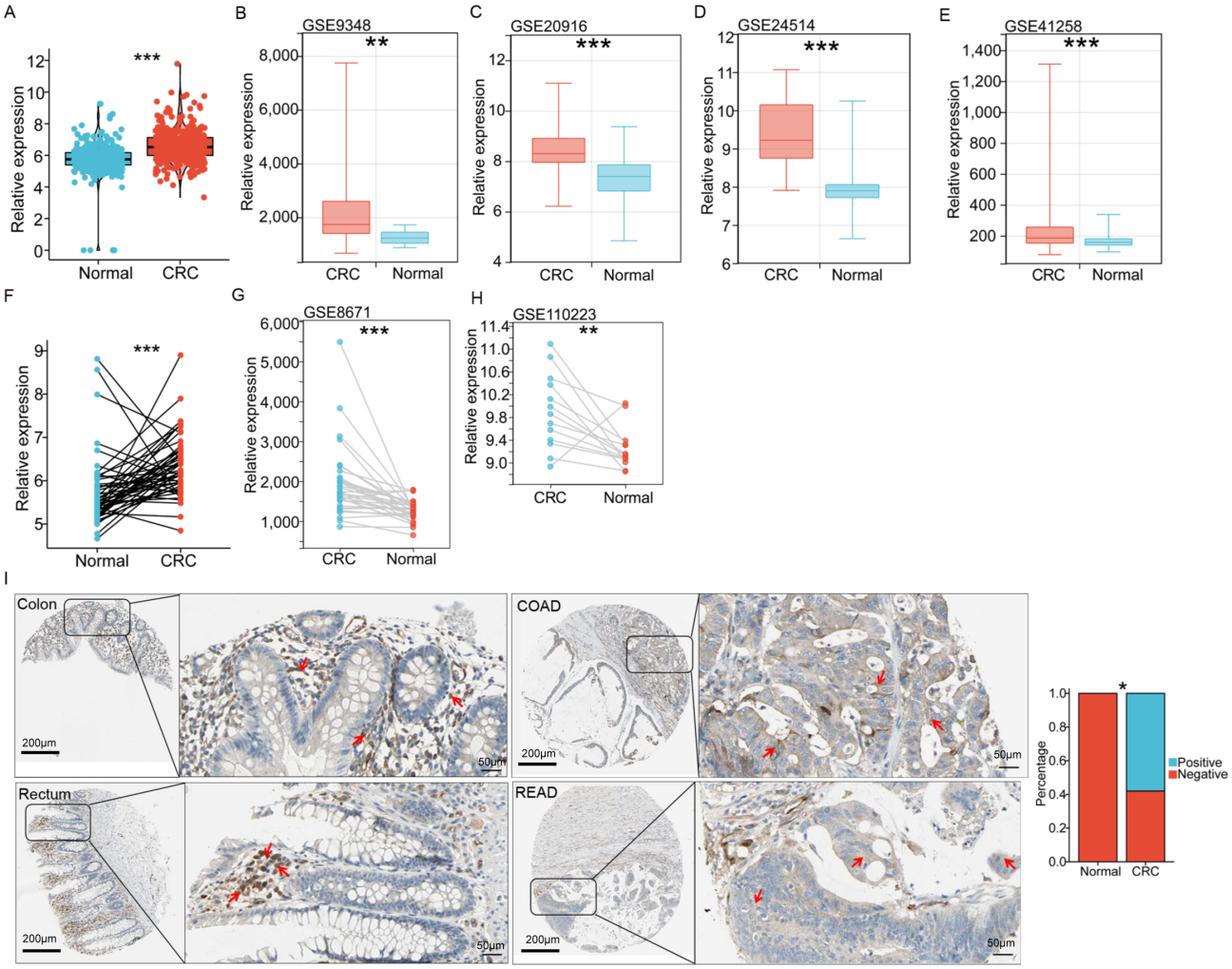

Fig. 2. *WARS1* expression and its prognostic significance in colorectal cancer (CRC). (A–E) Unpaired sample analysis of *WARS1* expression in TCGA, GSE9348, GSE20916, GSE24514, and GSE41258 cohorts. (F–H) Paired sample analysis of *WARS1* expression in TCGA, GSE8671, and GSE110223 cohorts. (I) *WARS1* expression in CRC and normal tissues detected by immunohistochemistry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar and magnification: 200 μm and 40× in the original drawing and 50 μm and 100× in the enlarged drawing. Red arrows: *WARS1*. COAD, colon adenocarcinoma; READ, rectum adenocarcinoma. N = 3.

regulation of the gene in 10 tumors, including COAD ($p < 0.05$; Fig. 1B). Proteomic analysis results showed significant upregulation of *WARS1* protein expression in 5 tumors, including COAD (all $p < 0.05$; Fig. 1C).

Various CRC datasets were analyzed to verify *WARS1* expression in CRC, revealing a considerable upregulation of *WARS1* mRNA levels ($p < 0.05$; Fig. 2A–H). Moreover, M category, pathologic stage, and histological type significantly influenced transcript levels ($p < 0.05$; Table 1).

Immunohistochemical data from the Human Protein Atlas indicated that *WARS1* protein was not expressed in endocrine cells, goblet cells, enterocytes, or their microvilli in normal colon tissues but was moderately expressed in the cytoplasm and cell membrane of COAD cells. Similarly, *WARS1* protein was not expressed in endocrine cells or enterocytes in normal rectum tissues but was expressed in the cytoplasm and cell membrane of READ cells. Statistical analysis revealed a significant increase in the positive rate of *WARS1* expression in CRC tissues compared to their corresponding normal tissues ($p < 0.05$; Fig. 2I).

Knocking Down WARS1 Inhibited CRC Cell Migration

The expression of *WARS1* was targeted for knockdown using three different shRNA pairs, and its inhibition was quantified via RT-qPCR and Western blotting to explore its cytological functions. The results demonstrated that all three shRNA pairs significantly inhibited *WARS1* expression ($p < 0.05$; Fig. 3A,B). Subsequently, the shRNA-1240 sequence was selected and packaged into a lentivirus to stably knock down the mRNA and protein expressions of *WARS1* ($p < 0.05$; Fig. 3C,D).

Wound-healing experiments revealed that cell migration was significantly inhibited after knocking down

Table 1. Correlation of *WARS1* with clinicopathological parameters in patients with colorectal cancer.

Characteristics	Low expression of *WARS1*	High expression of *WARS1*	*p* values	χ^2
n	322	322		
Pathologic T stage, n (%)			0.687	1.48
T1	12 (1.9%)	8 (1.2%)		
T2	52 (8.1%)	59 (9.2%)		
T3	220 (34.3%)	216 (33.7%)		
T4	35 (5.5%)	39 (6.1%)		
Pathologic N stage, n (%)			0.249	2.78
N0	175 (27.3%)	193 (30.2%)		
N1	85 (13.3%)	68 (10.6%)		
N2	60 (9.4%)	59 (9.2%)		
Pathologic M stage, n (%)			0.001	10.19
M0	222 (39.4%)	253 (44.9%)		
M1	58 (10.3%)	31 (5.5%)		
Pathologic stage, n (%)			0.039	8.39
Stage I	53 (8.5%)	58 (9.3%)		
Stage II	113 (18.1%)	125 (20.1%)		
Stage III	90 (14.4%)	94 (15.1%)		
Stage IV	58 (9.3%)	32 (5.1%)		
Gender, n (%)			0.937	0.01
Female	150 (23.3%)	151 (23.4%)		
Male	172 (26.7%)	171 (26.6%)		
Race, n (%)			0.093	4.75
Asian	7 (1.8%)	5 (1.3%)		
Black or African American	43 (10.9%)	26 (6.6%)		
White	151 (38.3%)	162 (41.1%)		
BMI, n (%)			0.648	0.21
$\leq$25	54 (16.4%)	53 (16.1%)		
>25	118 (35.9%)	104 (31.6%)		
Histological type, n (%)			0.001	10.41
Adenocarcinoma	290 (45.8%)	260 (41.1%)		
Mucinous adenocarcinoma	28 (4.4%)	55 (8.7%)		

The sample size is not 322 due to missing values. BMI, Body Mass Index.

WARS1 in RKO and HCT116 cells ($p < 0.05$; Fig. 3E,F). Moreover, the transwell assay also demonstrated significant suppression of cell migration following *WARS1* knockdown ($p < 0.05$; Fig. 3G–I).

Knocking Down WARS1 Inhibited CRC Cell Growth

The cell growth experiment demonstrated that the proliferation of both RKO and HCT116 cells was significantly suppressed when the level of *WARS1* was downregulated ($p < 0.05$; Fig. 4A,B). Additionally, results from the DepMap database confirmed that knocking out or knocking down the *WARS1* gene inhibited the growth of multiple CRC cell lines, including RKO and HCT116 (Fig. 4C,D).

Knocking Down WARS1 Arrested G1 Phase and Promoted Apoptosis

A flow cytometry experiment was conducted to elucidate the possible mechanism of low *WARS1* expression in inhibiting cell growth. The results demonstrated that knocking down *WARS1* significantly increased CRC cell apoptosis ($p < 0.05$; Fig. 4E) and also arrested the cells in the G1 phase of the cell cycle, affecting DNA replication ($p < 0.05$; Fig. 4F). These findings suggest that *WARS1* acts as a cancer-promoting gene in CRC cells.

Knocking Down WARS1 Reduced the Tumor Volume of Xenograft Tumors

A xenograft tumor experiment was conducted in nude mice to verify the tumor-promoting effect of *WARS1* on cell growth *in vivo*. Knocking down *WARS1* significantly inhibited tumor volumes ($p < 0.05$; Fig. 5A–C). Additionally, RT-qPCR and Western blotting validated a substantial downregulation of *WARS1* mRNA and protein in the sh*WARS1* group compared to the shCtrl group ($p < 0.05$; Fig. 5D,E).

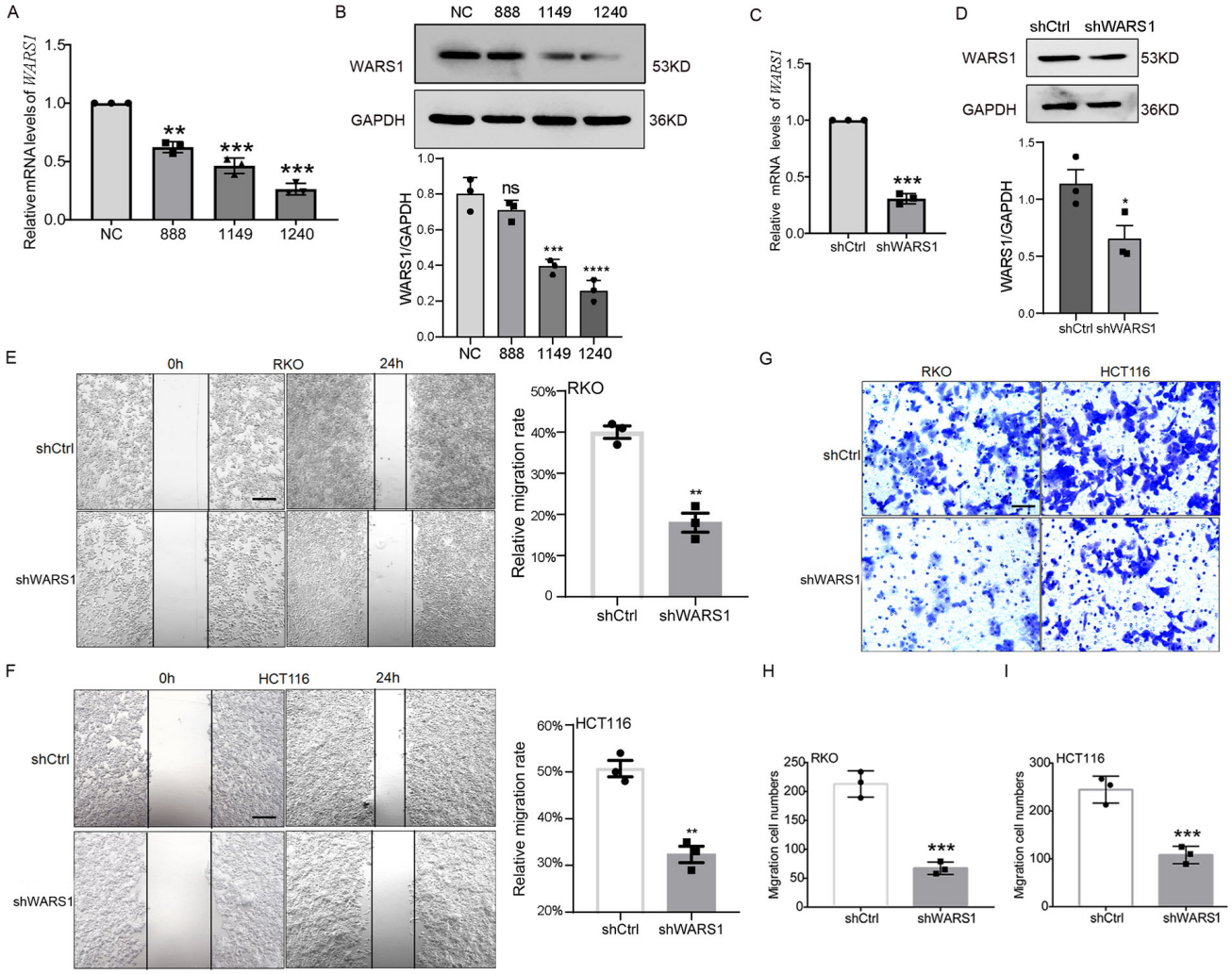

Fig. 3. The effect of *WARS1* on the migration and invasion of CRC cell lines. (A) RT-qPCR and (B) Western blotting were utilized to detect the knockdown effect of siRNAs on *WARS1*. (C) RT-qPCR and (D) Western blotting were used to detect the knockdown effect of shRNA on *WARS1*. (E,F) Wound healing and (G–I) transwell assays indicated that silencing *WARS1* expression inhibited the migration of RKO and HCT116 cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns $p > 0.05$. Scale bar: 50 µm. N = 3. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; NC, normal control; shCrtl, small hairpin control; sh*WARS1*, small hairpin *WARS1*.

The p53 Signaling Pathway Mediated Increased G1-Phase Arrest and Apoptosis during WARS1 Knockdown

Transcriptome sequencing analysis was conducted on the cell lines with *WARS1* knockdown to uncover the mechanisms by which downregulated *WARS1* expression promoted apoptosis and inhibited the cell cycle. A total of 1602 upregulated ($p < 0.05$) and 1275 downregulated genes ($p < 0.05$) were identified (Fig. 6A). The GSEA of these genes revealed that *WARS1* knockdown activated apoptosis pathways but inhibited cell cycle and DNA replication pathways (Fig. 6B). Additionally, the KEGG analysis indicated that the downregulated genes were primarily involved in the cell cycle and DNA replication (Fig. 6C). Conversely, the upregulated genes were mainly involved in the p53 signaling pathway and apoptosis (Fig. 6D). Differentially expressed genes (DEGs) in pathways such as the cell cycle and p53 pathways were visualized in heatmaps (Fig. 6E,F). Furthermore, RT-qPCR and Western blotting confirmed that the expression of *p53*, *TP53I3*, *BBC3*, tumor necrosis factor receptor superfamily *(TNFRSF)10A* and *B*, zinc finger matrin-type 3 (*ZMAT3*), and *CDKN1A* was significantly upregulated, while that of cyclin-dependent kinase 2 (*CDK2*), transcription factor Dp-1 (*TFDP1*), origin recognition complex subunit 1 (*ORC1*), *CDC6*, *CDC45*, minichromosome maintenance complex *(MCM) 2*, *3*, *4*, *5*, and *7* was considerably downregulated in the sh*WARS1* group ($p < 0.05$; Fig. 6G–K). Pifithrin-α (PFT-α), a widely used p53-specific inhibitor, effectively blocks the transcriptional activity of p53, thereby inhibiting downstream gene

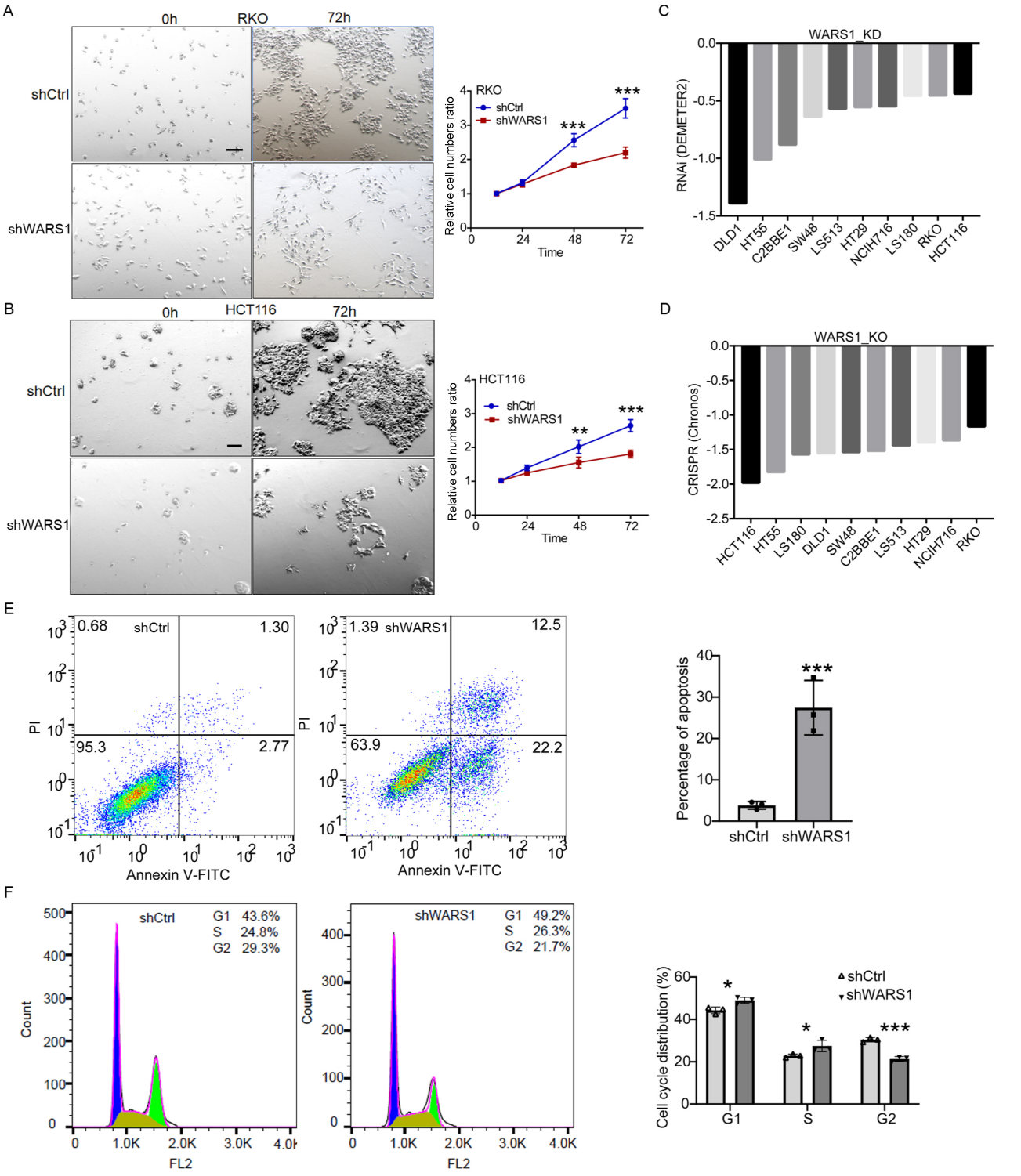

Fig. 4. The effect of *WARS1* on the growth of CRC cell lines. (A,B) Knockdown of *WARS1* inhibited the proliferative capacity of RKO and HCT116 cells. (C,D) Effect of *WARS1* knockout or knockdown on the growth of CRC cells based on the DepMap portal. (E,F) Flow cytometry showed that downregulation of *WARS1* expression promoted apoptosis and cell cycle arrest in CRC cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar: 50 µm. N = 3.

expression [22]. Remarkably, PFT-α prevented the activation of the apoptosis marker *CASP3* and downregulated the levels of *c-PARP1* and *CDKN1A* (all $p < 0.05$; Fig. 6L,M).

Discussion

The management of CRC remains a significant clinical challenge due to its late diagnosis and unfavorable prog-

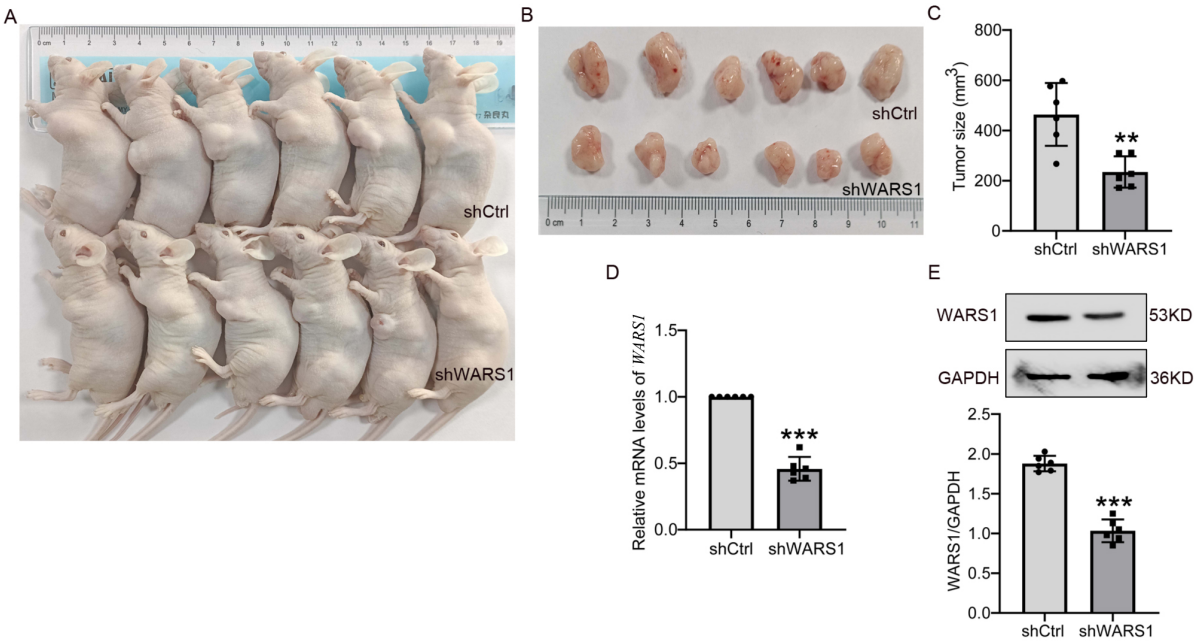

Fig. 5. The effect of *WARS1* on xenograft tumor growth. (A) Tumor formation in nude mice. Twelve nude mice were randomly divided into the experimental and control groups, with six mice in each group. 1.0×10^7 sh*WARS1* cells or control cells were injected subcutaneously into the left axilla of mice, respectively. The mice were photographed 28 days after the injection. (B,C) Downregulated *WARS1* expression inhibited the volume of xenograft tumors. (D) RT-qPCR and (E) Western blotting were employed to validate *WARS1* expression in xenograft tumors. ** $p < 0.01$, *** $p < 0.001$. N = 6.

nosis [6,23]. The absence of reliable targets could impede effective treatment and prognosis prediction for patients with advanced or metastatic CRC [4,8]. Therefore, the aim of this study was to explore the role of *WARS1* in CRC progression and its potential as a therapeutic target.

WARS1 plays diverse physiopathological roles in processes such as immune regulation, anti-angiogenesis, and tumor progression [9,24,25]. A study indicated that *WARS1* expression in gastric cancer tissue could serve as a valuable prognostic indicator for patients with locally advanced stomach cancer following successful surgical removal [26]. This study analyzed multiple datasets from TCGA and GEO databases, revealing a significant upregulation of *WARS1* mRNA levels in CRC tissues. Furthermore, immunohistochemical analysis detected *WARS1* expression in the cytoplasm and on the cell membrane in CRC samples, with a notably higher positive expression rate compared to normal tissues. Intriguingly, low *WARS1* expression correlated with inhibited CRC progression or recurrence, suggesting its potential as a therapeutic target for CRC patients. These findings align with those reported by Ghanipour A *et al.* [27] as they reported that low *WARS1* expression could not efficiently inhibit angiogenesis but could promote CRC recurrence or progression. Evidence suggests that *IFN-γ* triggers the expression and rapid secretion of *WARS1* from cells like macrophages and endothelial cells [28,29]. Once in the extracellular space, elastase cleaves the full-length *WARS1* protein into its T1 (residues 71–471) and T2 (residues 94–471) forms. Subsequently, the T2 form binds to VE-cadherin on endothelial cells, effectively inhibiting angiogenesis [9,30]. This mechanism potentially corroborates some of the findings in our study, reinforcing the pivotal role of *WARS1* in the pathological process of CRC.

Cytological outcomes revealed that the suppression of *WARS1* attenuated both proliferation and migratory capacities in CRC cell lines (RKO and HCT116). Additionally, flow cytometry analysis detected enhanced cell cycle arrest and apoptosis in the knockdown cells, suggesting a potential oncogenic role of *WARS1* in CRC cells. Noh KT *et al.* [13] reported that upregulating *WARS1* promotes naive $CD8^+$ T-cell proliferation, which could partially support the findings of this study. Furthermore, xenograft tumor experiments in the current study confirmed that *WARS1* knockdown inhibited tumor growth. In summary, *WARS1* may exhibit a dual function, as it demonstrates anti-angiogenic properties in the extracellular environment (e.g., T2 *WARS1*) [27,31], yet it could also promote the growth and migration of cancer cells. These discoveries underscore the crucial role of *WARS1* in tumorigenesis and progression; however, further exploration is needed to delineate its effects and molecular mechanisms in CRC.

Transcriptome sequencing revealed that the inhibition of *WARS1* upregulated mRNA and protein levels of p53 and its downstream key molecules, including BBC3, TP53I3, and CDKN1A. This led to the activation of the p53 signaling pathway, a classical tumor suppressor path-

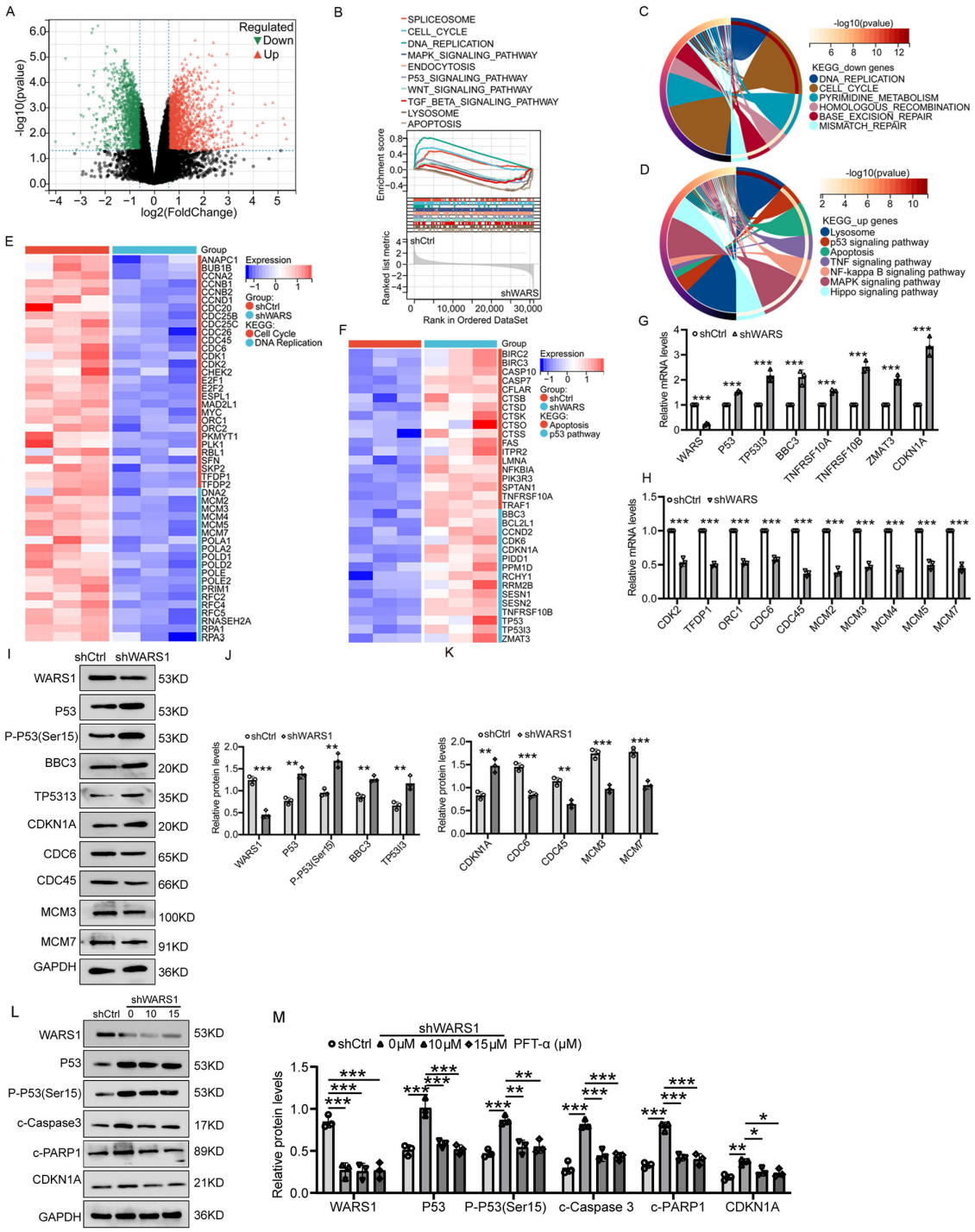

Fig. 6. Transcriptomic analysis to elucidate the role of *WARS1* in apoptosis and cell cycle of CRC cells. (A) Volcano map of the differentially expressed genes (DEGs) in *WARS1* knockdown. (B) Gene Set Enrichment Analysis (GSEA) of the *WARS1* gene. (C) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the downregulated genes, and (D) the upregulated genes. (E) Heatmap of the DEGs in cell cycle and DNA replication. (F) Heatmap of the DEGs in apoptosis and p53 signaling pathway. (G,H) RT-qPCR and (I–K) Western blotting detected DEGs in the cell cycle and p53 pathway. (L,M) Western blotting indicated that PFT-α blocked CASP3 activation and downregulated the expression of c-poly-ADP-ribose polymerases 1 (*PARP1*) and cyclin-dependent kinase inhibitor 1A (*CDKN1A*). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. *TP53I3*, tumor protein p53 inducible protein 3; *P-p53*, phosphorylation-protein53; *BBC3*, BCL2 binding component 3; *TNFRSF*, tumor necrosis factor receptor superfamily; *ZMAT3*, zinc finger matrin-type 3; *CDKN1A*, cyclin-dependent kinase inhibitor 1A; *CDK2*, cyclin-dependent kinase 2; *TFDP1*, transcription factor Dp-1; *ORC1*, origin recognition complex subunit 1; *CDC*, cell division cycle; *MCM*, minichromosome maintenance complex; *c-PARP1*, Cleaved poly-ADP-ribose polymerases 1.

way known to induce cell death, such as apoptosis, through both transcription-dependent and transcription-independent mechanisms [32–34]. Recovery experiments demonstrated that PFT-α [19] partially reversed the activation of the p53 pathway promoted by *WARS1* knockdown. This reduction in activation led to decreased levels of activated caspase 3 and its substrate c-PARP1, thereby inhibiting apoptosis. The inhibitor also restored the levels of CDKN1A, a transcriptional target of p53 responsible for mediating p53-induced G1 cell cycle arrest [35,36]. These findings collectively indicate that the p53 pathway partially mediates *WARS1* knockdown-induced cell apoptosis and cell cycle inhibition.

This study confirmed the overexpression of *WARS1* in CRC and demonstrated that its downregulation effectively inhibited the progression and recurrence of CRC. Interestingly, *WARS1* exhibited dual functions; while spliced *WARS1* inhibited angiogenesis in the extracellular environment, it also promoted cell proliferation in CRC cells. The p53 signaling pathway partially mediated the apoptosis and cell cycle inhibition induced by *WARS1* knockdown. Future research will systematically address the limitations of this study, employing a more robust theoretical basis to support the development of drugs targeting *WARS1*. Further investigations will focus on elucidating the molecular mechanisms by which *WARS1* regulates the p53 pathway in cancer cells and exploring whether extracellular *WARS1* protein exerts additional regulatory functions in the tumor microenvironment.

Conclusion

This study revealed that *WARS1* was highly expressed in CRC and that its low expression correlated with an increased risk of CRC progression and recurrence. *WARS1* was found to promote colorectal carcinogenesis and progression by enhancing cell proliferation and migration while inhibiting apoptosis. Knockdown of *WARS1* resulted in increased apoptosis and inhibited cell cycle progression through activation of the p53 signaling pathway. These findings validate *WARS1* as a potential therapeutic target and lay a solid foundation for the development of therapeutic agents targeting *WARS1* in CRC.

Availability of Data and Materials

All datasets generated for this study are included in the article/Supplementary Material. Clinical data of CRC patients and the corresponding RNA expression profiles were obtained from TCGA through the UCSC Xena website (https://xenabrowser.net/datapages/). Multi-source datasets were retrieved from the publicly available GEO database (https://www.ncbi.nlm.nih.gov/). The protein levels of *WARS1* were assessed with the UALCAN (https://ualcan.path.uab.edu/index.html) and Human Protein Atlas database (https://www.proteinatlas.org/).

Author Contributions

YZ and XND designed the study, MH and HRZ downloaded, processed, and analyzed the data, MH performed experiments, YZ, XND, and HRZ drafted the manuscript and MH and YZ contributed to the important editorial changes in the manuscript. All authors reviewed and approved the final manuscript. All authors agreed to be 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.

Ethics Approval and Consent to Participate

All experimental protocols adhered to the Standard Operating Procedures established by the Jiamusi University Biological and Medical Ethics Committee (JMSU-2023112701).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537192.10.

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