


# Up-frameshift Protein 1 Promotes Tumor Progression by Regulating Apoptosis and Epithelial–Mesenchymal Transition of Colorectal Cancer

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## Abstract

**Background:** Recently, accumulating evidence confirmed that up-frameshift protein 1 (UPFI) was aberrantly expressed in various cancers. However, the molecular mechanism mediated by UPFI underlying colorectal carcinogenesis remains unclear. **Method:** Immunohistochemistry (IHC) and quantitative real-time polymerase chain reaction analysis were used to determine the expression level of UPFI in colorectal cancer (CRC) tissues. CCK-8, EdU, transwell assay, and flow cytometry were performed to investigate the biological significance of UPFI. Epithelial–mesenchymal transition (EMT) and apoptosis associated markers were detected by western blotting. **Results:** We found that UPFI expression was upregulated in CRC tissues and cell lines. Clinical analysis revealed that high UPFI expression was positively correlated with advanced stage, lymph node metastasis and shorter survival. Knockdown of UPFI suppressed cell proliferation and cell cycle progression. Functionally, UPFI promotes tumor metastasis by inducing epithelial to mesenchymal transition. Further investigations revealed that knockdown of UPFI promoted apoptosis through triggering DNA damage. **Conclusions:** Taken together, this research revealed that UPFI plays an oncogenic role in CRC via regulating EMT and apoptosis and may be a potential therapeutic target for CRC.

## Keywords

colorectal cancer, up-frameshift protein 1, epithelial–mesenchymal transition, DNA damage, apoptosis

## Abbreviations

ATR, ataxia telangiectasia mutated and RAD3-related; BAX, bcl-2-associated X protein; BCL-2, B-cell lymphoma-2; CRC, colorectal cancer; DSB, double strand breaks; EMT, epithelial–mesenchymal transition; FACS, flow cytometry analysis; HCC, hepatocellular cancer; lncRNA, long noncoding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NMD, nonsense-mediated mRNA decay; OS, overall survival; PTC, premature termination codons; SMG-1, suppressor with morphogenetic effect on genitalia-1; SNHG6, small nucleolar RNAs host gene 6; TGF- $\beta$ , transforming growth factor beta; UPF2/UPF3, up-frameshift protein 2/3; ZFX, zinc finger protein X; 53BP1, p53-binding protein 1.

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## Introduction

Colorectal cancer (CRC) is the third frequent malignancy and is the second leading cause of cancer-related deaths worldwide.<sup>1</sup> Incident rates of CRC rose by 2.2% annually overall.<sup>2</sup> Although screening and treatments of CRC get advanced recently, majority of patients hold a poor prognosis.<sup>2</sup> Therefore, better understanding of potential prognosis biomarkers and therapeutic targets is needed to improve survival of CRC patients.

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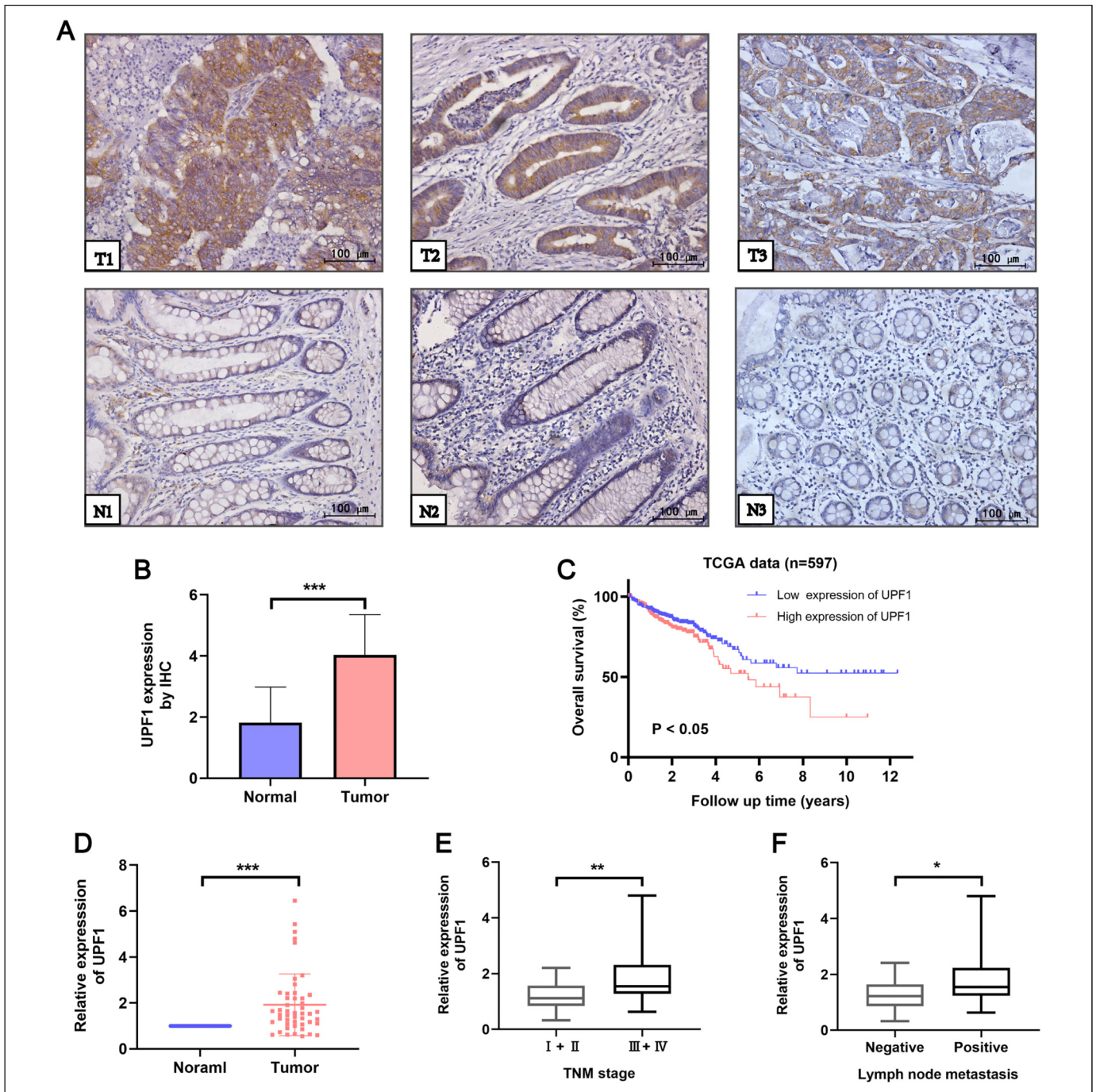
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**Figure 1.** UPF1 expression is upregulated in CRC tissues compared to adjacent normal tissues. (A,B) The protein expression level of UPF1 was detected in 48 pairs of CRC and adjacent normal tissues via IHC staining (200 $\times$ ). (C) Kaplan-Meier curves showed that CRC patients with higher expression of UPF1 had worse overall survival ( $P < .05$ , log-rank test). (D) The mRNA expression level of UPF1 was measured in 48 pairs of CRC and adjacent normal tissues via RT-qPCR. (E) Correlation between UPF1 mRNA expression level in CRC patients and clinical TNM stage. (F) Correlation between UPF1 mRNA expression level in CRC patients and lymph node metastasis. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ .

Up-frameshift protein 1 (UPF1), located at chromosome 19p13.2-p13.11, is a highly conserved and ubiquitously expressed phosphoprotein with RNA/DNA-dependent ATPase and RNA helicase activity.<sup>3</sup> Depending on its ATPase and helicase activities,<sup>4,5</sup> UPF1 serves as the core regulator of nonsense-mediated mRNA decay machinery, which is

a RNA surveillance pathway protecting cells from damage by premature termination codons.<sup>6</sup> Also, UPF1 is involved in cell proliferation and differentiation.<sup>7,8</sup> In addition, UPF1 is required for S phase progression and genome stability, which is essential for DNA damage repair.<sup>9</sup> Previous studies have clarified the exact activities of UPF1 in hepatocellular cancer<sup>10</sup> and

**Table 1.** Relation Between Clinicopathological Features and UPF1 Expression in CRC Patients (n = 48).

Parameters	Cases	UPF1 expression		P value
		Low (n = 25)	High (n = 23)	
Gender				
Female	16	6	10	.153
Male	32	19	13	
Age (years)				
< 60	17	7	10	.263
≥60	31	18	13	
Stage				
I + II	20	15	5	.007*
III + IV	28	10	18	
Tumor size				
< 5 cm	25	14	11	.571
≥5 cm	23	11	12	
Lymphatic status				
positive	26	9	17	.008*
negative	22	16	6	

\*P&lt;.05

gastric cancer,<sup>10</sup> however, UPF1 exact biological activities in human CRC are poorly understood.

DNA damage is the well-known trigger of the intrinsic apoptosis pathway. Signaling to intrinsic apoptosis due to DNA damage stimuli the release of cytochrome C and activate BCL-2 family, promoting the formation of apoptosome.<sup>11,12</sup> Then, procaspase 9 is activated, which in turn activates the executioner apoptosis cleaved caspase 3, leading to the death of cells.<sup>13</sup> Double strand breaks (DSB) are widely recognized to be among the most deadly forms of DNA damage, causing genomic instability.<sup>14</sup> H2AX is considered to be the hallmark of DNA damage and DSB, its phosphorylation is tightly linked to DNA damage repairs.<sup>15</sup> According to reports, the expression of H2AX is increased in UPF1-depleted cells.<sup>9</sup> These observations arise our interest to explore whether and how UPF1 and H2AX are involved in tumorigenesis of CRC and the underlying mechanism driving the relationships with apoptosis.

In this study, we demonstrated that UPF1 was significantly upregulated in CRC tissues compared with adjacent normal tissues. Subsequently, we investigated the contribution of UPF1 to the proliferation and metastasis of CRC as well as its effect on DNA damage and apoptosis, which provides a new sight for clinical diagnosis and treatment of CRC.

## Materials and Methods

### Colorectal Clinical Specimens and Cell Lines

Forty-eight pairs of CRC specimens and paired adjacent non-cancer tissues were collected from patients of The First Affiliated Hospital of Shantou University Medical College. Tumor staging relies on eighth American Joint Committee on Cancer Tumor-node-metastasis classification (AJCC TNM-8).

All the patients who participated in this study had signed the written informed consent voluntarily. The present study was approved by the Medical Ethics Committee of The First Affiliated Hospital of Shantou University Medical College.

The FHC human normal colorectal epithelial cell line and 4 human CRC cell lines (SW480, SW620, RKO, Caco-2) were cultured with DMEM supplemented with 10% fetal bovine serum (Gibco, Australia origin) in a water-saturated environment at 37 °C with 5% CO<sub>2</sub>. All the cell lines (FHC, SW480, SW620, RKO, Caco-2) were donated by General surgery laboratory of Southern Medical University.

### Immunohistochemistry

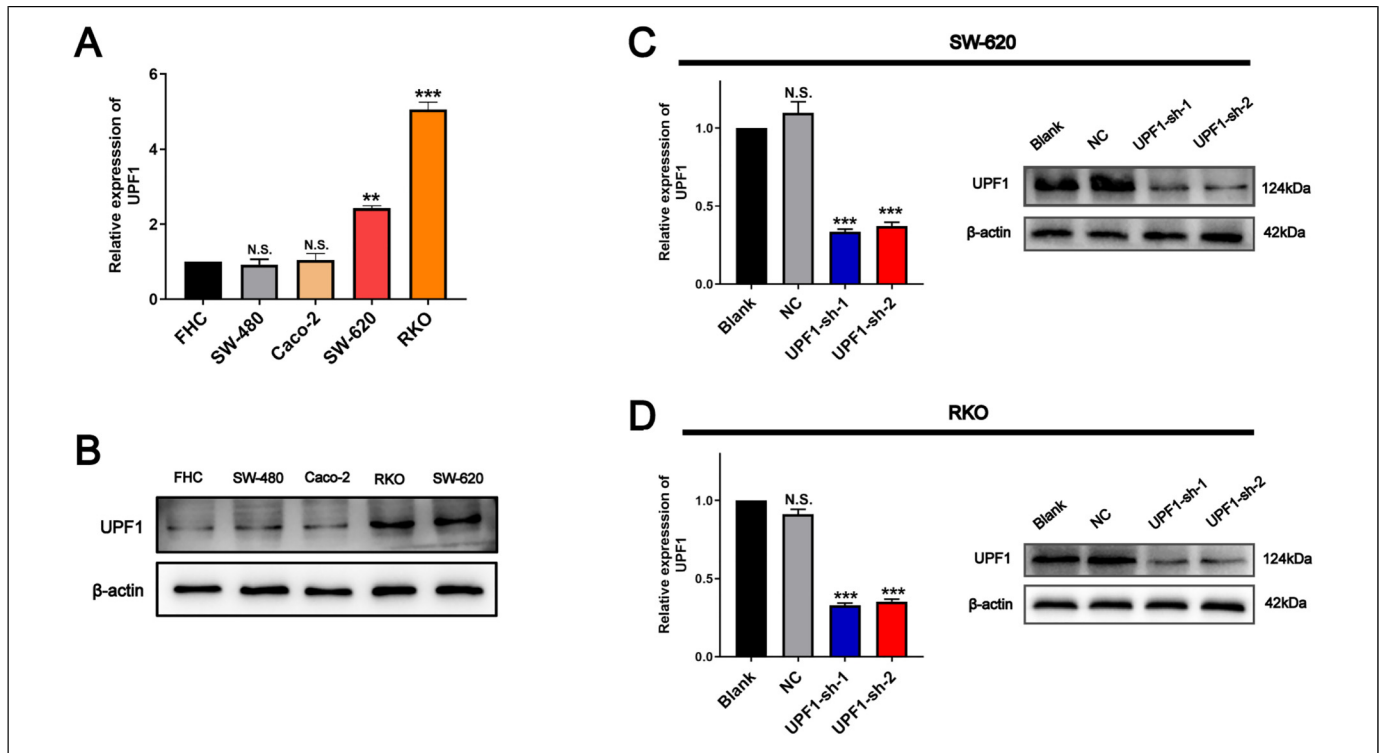
Immunohistochemical staining of 48 pairs of CRC tissues and paired normal tissues was performed on 4 μm thick paraffin sections. All the procedures were referred to manufacturer's protocol using an UltraSensitive™ S-P kit and DAB (Maixinbio, China). Antibody UPF1(dilution of 1:10000, ab109363, abcam) was applied. Cytoplasm stained with brownish yellow is considered positive. The staining paraffin sections were imaged with microscope (200× magnification). Image J software was used to evaluate the immunohistochemistry (IHC) staining. The percent positively stained areas were scored as "1" (0-25%), "2" (26-50%), and "3" (≥50%). The staining intensity was scored as "0" (no staining), "1" (weak), "2" (moderate), and "3" (strong). Then we calculated the sum of the 2 scores. The total score ≤3 was defined as negative expression, while the total score ≥4 was defined as positive expression. All specimens were evaluated by 2 pathologists who were blinded to the patient identity and clinical outcome.

### Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from fresh tissue using EasyPure RNA Kit (TransGen) and was reverse-transcribed into cDNA using a PrimeScript RT Master Mix (TaKaRa). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using SYBR Premix Ex Taq II Kit (TaKaRa) on CFX Connect. β-actin was used as an internal control. The relative expression was evaluated using 2<sup>-ΔΔct</sup> method. The primers used are as follows: β-actin (5'-TGGCACCCAGCACAATGAA-3'; 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'); UPF1 (5'-A GAGCCTCATGCAGTTCAGCAA-3'; 5'-GGCATCATACA TGGCTGTGGTC-3').

### Plasmid Constructions and Transfection

UPF1-shRNAs were designed and synthesized by GeneCopoeia. The sequence of shRNAs used are as follows UPF1-shRNA #1: 5'-GCAGCCACATTGTAATCACC-3'; UPF1-shRNA #2: 5'-GCGTGGTTTACTGTAATACCA-3'; UPF1-shRNA #3: 5'-CCTATTACACGAAGGACCTCC-3'. CRC cell lines SW620 and RKO were transfected using Lipofectamine 3000 Transfection Reagent (Thermo Fisher).



**Figure 2.** UPF1 is upregulated in CRC cell lines. (A, B) UPF1 expression level was upregulated in CRC cell lines, especially in RKO and SW620. (C, D) Knockdown efficiency of UPF1 was confirmed via RT-qPCR. N.S., not significant.  $**P < .01$ ,  $***P < .001$ .

At 48 h after transfection, qRT-PCR and western blotting were used to confirm the expression of UPF1.

### Cell Proliferation Assay

CCK-8 assay was used to examine cell proliferation. SW620 and RKO cell lines with UPF1 knockdown were seeded on the 96-well plates at a concentration of  $3 \times 10^3$  of RKO while  $5 \times 10^3$  of SW620 per well with 100  $\mu$ l DMEM containing 10% CCK-8 solution (CCK-8, Dojindo). Then, each well was cultured for 24, 48, 72, and 96 h. Cells with CCK-8 reagent were incubated for 4 h at 37  $^{\circ}$ C. Finally, the absorbance value (OD) of 450 nm was read on the microplate reader (Bio-Rad).

EdU staining was also used to assess cell proliferation. CRC cells seeded in each well of 24-well plates at a density of  $4 \times 10^4$  cells/well of RKO while  $6 \times 10^4$  cells/well of SW620 were stained using Meilun EdU cell proliferation Kit with Alexa Fluor 488 (Meilun, Dalian) referring to manufacturer's protocol. Lastly, the cells were imaged with inverted microscope (100 $\times$  magnification).

### Transwell Assay

The migration and invasion of cells were assessed using transwell chamber (0.8 mm) with Matrigel-coated or not. Transfected RKO cells ( $4 \times 10^4$ ) and SW620 cells ( $6 \times 10^4$ ) were separately seeded in upper chamber with 100  $\mu$ l serum-free medium. The lower chamber was filled with 500  $\mu$ l medium containing 20% FBS. After 48 h, cells trapped in the

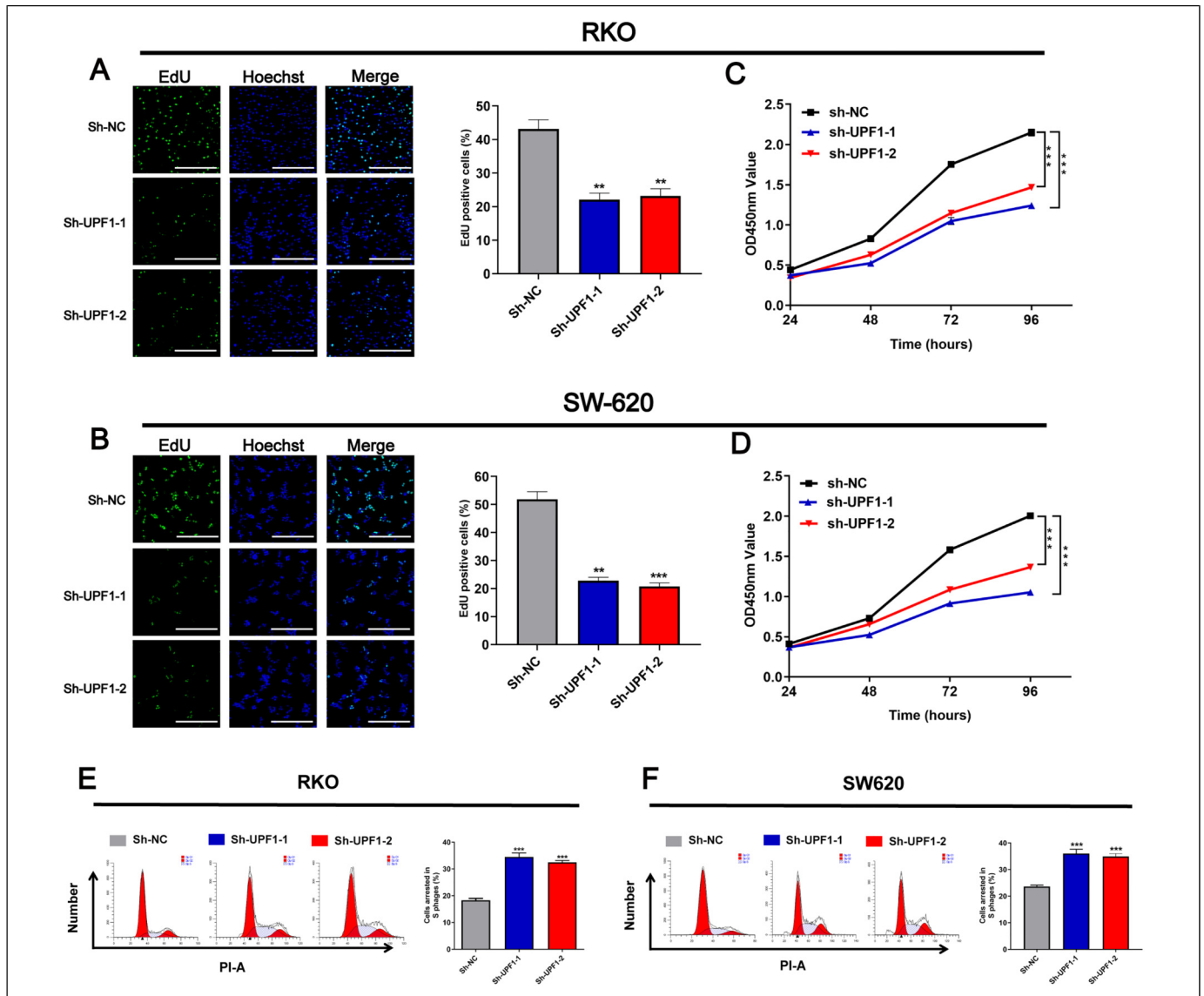
chamber membrane surface were fixed with 4% paraformaldehyde for 15 min, stained using 0.1% crystal violet for 15 min and imaged with microscope (400 $\times$  magnification). Migrated and invaded cells of each sample were counted in 5 random fields for statistical analysis.

### Flow Cytometric Analyses

Transfected RKO cells and SW620 cells were collected 48 h after transfection and then stained using cell cycle detection kit (MultiSciences) following the manufacturer's instructions. The percentage of cells in the cell cycle stages was analyzed by a FACSCanto II flow cytometer (BD Biosciences). For the cell apoptosis assays, transfected cells were analyzed using Annexin V-FITC/PI apoptosis detection kit (4A Bio) according to the manufacturer's instructions, cell apoptosis rate was then evaluated by FACSCanto II flow cytometer.

### Western Blotting

Total proteins in cells were extracted by radio-immunoprecipitation assay buffer (RIPA) and the proteins concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). The targeted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking and 12% separating gels) and transferred onto PVDF membranes which were blocked with 5% bovine serum albumin (BSA) solution for 1 h. Then the



**Figure 3.** Knockdown UPF1 inhibited cell proliferation and cell cycle of CRC cell lines. (A, B) EdU staining showed that UPF1 depletion inhibited cell growth in RKO and SW620 cells (100 $\times$ ). Scale bar = 200  $\mu$ m. (C, D) CCK8 assay showed that UPF1 depletion inhibited cell growth in RKO and SW620 cells. (E, F) Flow cytometry analysis showing the proportion of CRC cells in the G1, S, and G2/M phases when UPF1 was silenced in RKO and SW620 cells. \*\* $P < .01$ , \*\*\* $P < .001$ .

polyvinylidene fluoride (PVDF) membranes with targeted proteins were incubated with primary antibody including UPF1 (dilution of 1:10000, ab109363, abcam),  $\beta$ -actin (dilution of 1:1000, 4ab000001, 4A Biotech), E-cadherin (dilution of 1:1000, #3195, cell signaling), Vimentin (dilution of 1:1000, #5741, cell signaling), H2AX (dilution of 1:1000, #7631, cell signaling), Bax(dilution of 1:1000, #5023, cell signaling), Bcl-2 (dilution of 1:1000, Ab32124, abcam), Caspase3 (dilution of 1:1000, #14220, cell signaling), Cleaved Caspase3 (dilution of 1:1000, #9661, cell signaling), and paired secondary antibody goat anti-rabbit/mouse IgG H&L (HRP) (dilution of 1:2000, SA00001-1/ SA00001-2, Proteintech). Finally, the proteins bands were detected using ECL chemiluminescence system.

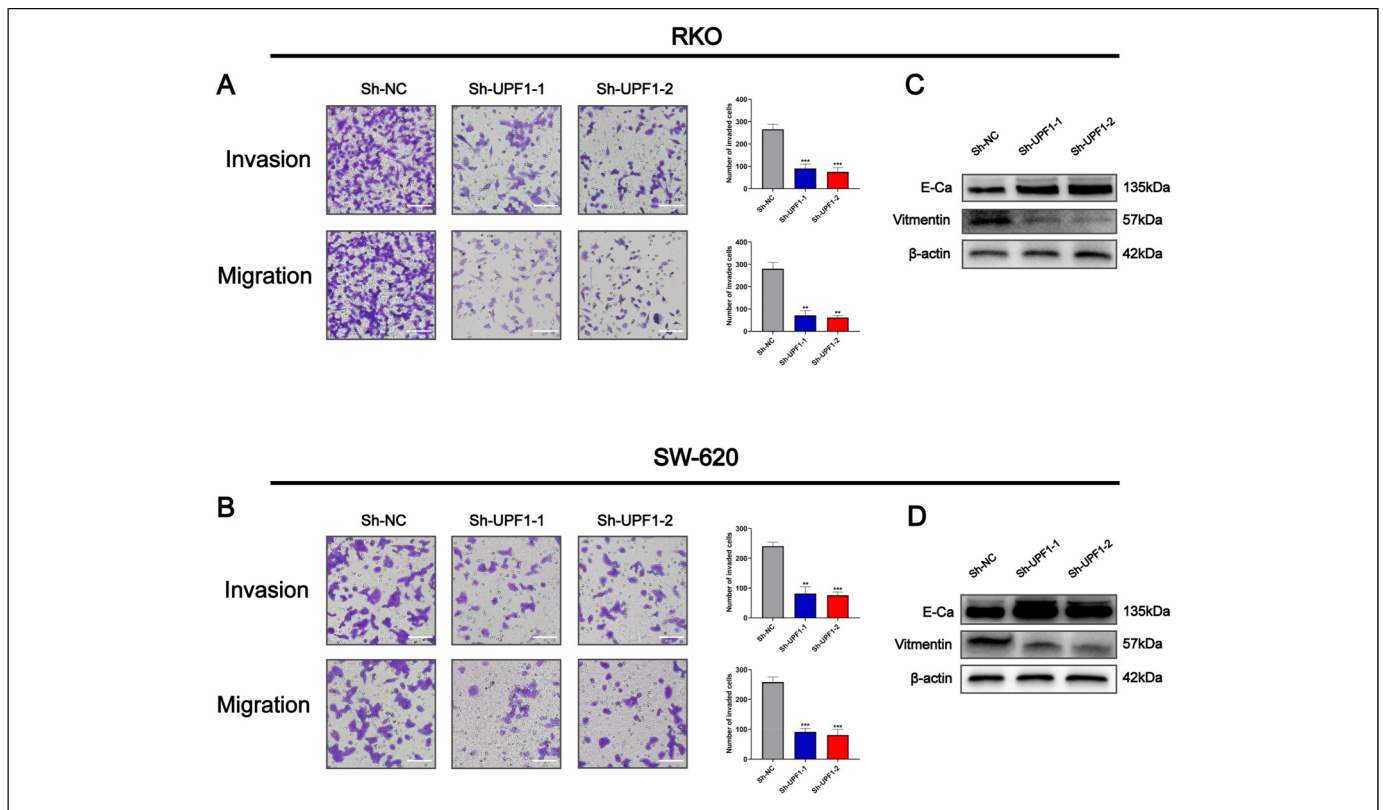
### Statistical Analysis

All experiments were repeated at least 3 times and the data were expressed as the means  $\pm$  standard deviation. The data were evaluated using Student's  $t$ -test and  $\chi^2$ -test. And  $P < .05$  was considered to be significant. Statistical graphs were performed using GraphPad Prism software.

### Results

#### UPF1 Expression is Significantly Upregulated in CRC and Correlates with Patients' Prognosis

To evaluate the expression and clinical significances of UPF1 in CRC, we first measured its expression level in 48 CRC patients



**Figure 4.** Knockdown UPF1 inhibited cell migration, invasion, and EMT in CRC cell lines. (A, B) The ability of cell migration and invasion was inhibited when UPF1 was silenced in RKO and SW620 cells (400×). Scale bar = 200 μm. (C, D) UPF1 depletion upregulated E-cadherin expression while downregulated the expression of N-cadherin and vimentin. \*\* $P < .01$ , \*\*\* $P < .001$ .

using IHC staining. The results showed that UPF1 was significantly upregulated in CRC tissues compared to adjacent normal tissues (Figure 1A and B). qRT-PCR was further performed to examine the expression of UPF1 in 48 CRC patients. As shown in Figure 1D, UPF1 expression was higher in CRC tissues than in adjacent normal mucosa. Next, we separated these patients into UPF1 high ( $n = 23$ ) and low ( $n = 25$ ) group according to median value. Clinical data showed that aberrant expression of UPF1 was positively associated with the positive lymph node metastasis and TMN stage (Figure 1E and F, Table 1). Then, we downloaded the data containing 597 patients from the TCGA-COAD dataset. Kaplan-Meier and log-rank test analyses showed that patients with higher expression of UPF1 had significantly shorter overall survival (OS) (Figure 1C).

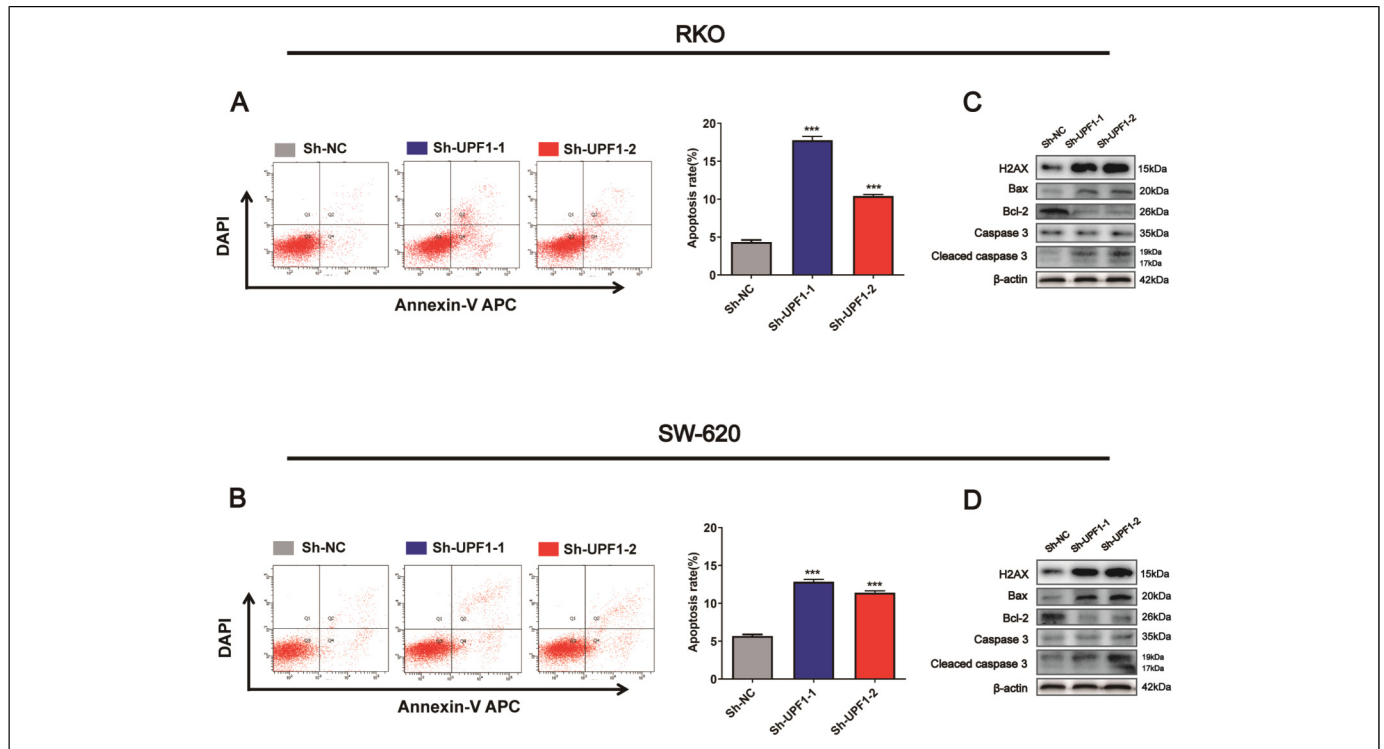
#### Knockdown of UPF1 Inhibits CRC Cell Proliferation and Cell Cycle Progression

Similarly, qRT-PCR showed that UPF1 expression was higher in a group of CRC cell lines compared with FHC cell line, which is an immortalized normal human colorectal cell line (Figure 2A). Western blotting further proved the higher expression of UPF1 in CRC cell lines (Figure 2B), especially in SW620 and RKO. Therefore, these 2 cell lines were employed to confirm the oncogene role of UPF1 in vitro. To further

investigate the biological function of UPF1, we knocked down UPF1 in SW620 and RKO cells by transfecting specific shRNA against UPF1. The results showed that the mRNA and protein levels were significantly reduced in 2 CRC cells transfected with sh-UPF1-1 and sh-UPF1-2 (Figure 2C and D). CCK8 and EdU proliferation assays revealed that silencing UPF1 inhibited cell proliferation relative to the negative control (NC) groups (Figure 3A-D). In addition, the cell cycle assays showed that silencing of UPF1 increased the proportion of cells in S phase (Figure 3E and F).

#### Knockdown of UPF1 Inhibits Cell Migration, Invasion, and EMT in CRC Cells

To further examine the effects of UPF1 on tumor metastasis, we evaluated the biological activity of UPF1 in regulating migration and invasion through transwell assays. As shown in Figure 4, silencing of UPF1 repressed the migration and invasion abilities of SW620 and RKO cells (Figure 4A and B). As EMT is well known for tumor metastasis, the protein level of EMT markers was evaluated by western blotting. Silencing of UPF1 increased the expression of epithelial marker E-cadherin, while repressed the expression of mesenchymal marker vimentin (Figure 4C and D). Taken together, our



**Figure 5.** Knockdown UPF1 promoted cell apoptosis by triggering DNA damage. (A, B) Knockdown UPF1 induced apoptosis in RKO and SW320. (C, D) Western blotting analysis of H2AX protein expression and apoptosis marker proteins following transfection of UPF1-shRNA. \*\*\* $P < .001$ .

results revealed that knockdown of UPF1 inhibits cell migration, invasion, and EMT in CRC.

### Knockdown of UPF1 Promotes Apoptosis by Inducing DNA Damage in CRC

According to the study by Azzalin and his colleagues, UPF1 was essential to DNA stability, and DNA damage might be triggered by silencing of UPF1.<sup>16</sup> Previous studies had demonstrated that UPF1 was involved in apoptosis regulation,<sup>10,11</sup> however, whether UPF1 could regulate apoptosis through triggering DNA damage promotes us to explore the underlying mechanism in CRC cells. As shown, the expression of DNA damage marker H2AX was significantly upregulated when UPF1 was silenced in SW620 and RKO cells (Figure 5C and D). Then, apoptosis flow cytometry analysis (FACS) showed that UPF1-knockdown induced apoptosis in the 2 cells (Figure 5A and B). We also evaluated the expression of apoptosis markers in cells transfected with UPF1-shRNA. The results showed that knockdown of UPF1 increased the pro-apoptosis marker BAX protein expression while decreased the anti-apoptosis marker Bcl-2 protein expression. In addition, cleaved caspase 3 were upregulated in UPF1-knockdown CRC cells compared to control cells, while the protein levels of procaspase 3 were not significantly altered (Figure 5C and D).

In addition, DNA mismatch repair (MMR) is also implicated in apoptosis after DNA damage.<sup>17</sup> In order to explore the

relationship between UPF1 and MMR proteins, we performed analysis using GEPIA database. As shown in Supplemental Figure S1, expression of UPF1 was positively correlated with MMR proteins including MLH1, MSH2, MSH6, and PMS2 (Supplemental Fig. S1 A-D). Therefore, we concluded that knockdown of UPF1 caused damage to DNA, and consequently promoted apoptosis in CRC cells.

### Discussion

Our study showed that UPF1 was upregulated in CRC tissues and cell line. Moreover, high expression promoted cell growth and metastasis of malignant CRC cells, which agreed with the clinicopathological parameter. The expression of UPF1 was associated closely with TMN stages and lymphatic penetration in CRC. Therefore, our data demonstrated that UPF1 has a potential to act as a prognostic biomarker for CRC. However, more samples should be included to determine the clinical value of UPF1 in CRC.

EMT is recognized to be a crucial mechanism that plays an important role in cancer invasion and metastasis.<sup>16</sup> In this process, cells which lose epithelial characteristics gain migratory and invasive properties.<sup>18</sup> Our study elucidated that knockdown of UPF1 increased expression E-cadherin with reduced expression of Vimentin. These data suggested that UPF1 could activate process of EMT in CRC, leading to the enhanced metastasis. Tumor budding is a highly aggressive

subpopulation of tumor cells with migratory and invasive capacities.<sup>19</sup> According to researches of Gurzu et al., EMT was associated with the tumor-budding degree in CRC. Budding degree could be used to evaluate EMT in CRC.<sup>20,21</sup> However, whether UPF1 was correlated with tumor budding and the mechanism behind EMT are unclear, which should be further investigated in the following research.

Previous studies have demonstrated that UPF1 was essential in various biological processes including embryonic and brain development.<sup>22</sup> In *Drosophila melanogaster*, UPF1 loss-of-function suppressed cell growth and triggered apoptosis.<sup>23</sup> However, dysfunction of UPF1 also contributed in tumorigenesis through regulating apoptosis. Actually, how UPF1 inducing apoptosis still remains unclear. As Azzalin and his colleagues mentioned, UPF1 was necessary to genome stability and UPF1-depletion might trigger DNA damage.<sup>9,24</sup> In our study, we found that UPF1 silencing induced DNA damage response and increased the proportion of cells in S phase. Intriguingly, apoptotic rate in UPF1-depleted cell was significantly increased. It is known that intrinsic apoptosis pathway signal could arise from DNA damage.<sup>12</sup> Taken together, we concluded that UPF1-depletion regulated apoptosis through inducing DNA damage. However, more downstream functional experiments still determine to be clarified in the following research.

Actually, the function of UPF1 is required for genome stability and is dual in tumorigenesis. Chang et al. clarified that UPF1 could suppress HCC invasion, migration, and proliferation through activating the TGF- $\beta$ /Smad pathway by eliminating lncRNA SNHG6.<sup>25</sup> Whereas, Shao and his colleagues demonstrated that UPF1 increased cell viability and promoted metastasis via enhancing the stability of linc-00313 in glioma.<sup>26</sup> Our results showed that knockdown of UPF1 suppressed the malignant biological behaviors of CRC cells via triggering DNA damage. It seems that UPF1 phosphorylated by different kinases lead to different DNA or RNA metabolism processes. On the one hand, phosphorylated by SMG-1, UPF1 recognizes targeted mRNA and then works with UPF2/UPF3 to trigger the decay of aberrant RNA.<sup>27,28</sup> On the other, modified by ATR phosphorylation, UPF1 is essential in DNA stability.<sup>24</sup> Herein, we elucidated regulatory roles of UPF1 in CRC. However, whether DNA damage caused by UPF1 knockdown was induced by ATR phosphorylation in CRC cells should be further investigated in the future.

## Conclusion

Our study suggested that the upregulation of UPF1 in tumor tissues was significantly correlated with poor prognosis. Furthermore, UPF1 depletion was involved in CRC progression through activated apoptosis and EMT progression. All the results indicated that UPF1 played an important role in CRC carcinogenesis and may serve as a potential target for CRC diagnosis and therapy.

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## Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Ethics approval and informed consent

The present study was authorized by the Research Ethics Review Board of The First Affiliated Hospital of Shantou University Medical College (No. 2020-102). All the patients who enrolled in this research had signed the written informed consent voluntarily.

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## Supplemental Material

Supplemental material for this article is available online.

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