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Silence of ATAD2 inhibits proliferation of colorectal carcinoma *via* the Rb-E2F1 signaling

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Abstract. – OBJECTIVE: This study aims to clarify the potential function of ATAD2 (ATPase family, AAA domain containing 2) in regulating proliferative and apoptotic abilities of colorectal carcinoma (CRC).

PATIENTS AND METHODS: ATAD2 levels in CRC specimens and cell lines were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Overall survival in CRC patients with high or low level of ATAD2 was assessed by Kaplan-Meier method. The correlation between ATAD2 level and clinical characteristics of CRC patients was analyzed by χ^2 test. Univariable and multivariable Cox regression models were generated to illustrate potential risk factors for the overall survival of CRC. After knockdown of ATAD2 in SW620 cells, relative levels of Cyclin D1, ppRb, pRb, E2F1, Cyclin E and cleaved Caspase 3 were detected by Western blot. Regulatory effects of ATAD2 on viability, clonality, cell cycle distribution, and apoptosis in SW620 and HCT15 cells were examined by a series of functional experiments.

RESULTS: Upregulated ATAD2 in CRC was correlated to tumor size, tumor node metastasis (TNM) staging, and histological classification of CRC. High level of ATAD2 predicted poor prognosis in CRC patients. Cox regression test suggested that ATAD2 level, tumor size, TNM staging and histological classification were independent factors influencing overall survival in CRC. Knockdown of ATAD2 reduced viability and clonality in SW620 and HCT15 cells. In addition, cell cycle was arrested in G1 phase and apoptosis was stimulated in CRC cells with ATAD2 knockdown. In SW620 cells transfected with ATAD2 shRNA, protein levels of Cyclin D1, ppRb, E2F1 and Cyclin E were downregulated, and cleaved Caspase 3 was upregulated.

CONCLUSIONS: ATAD2 is upregulated in CRC tissues and correlated to poor prognosis of CRC patients. It exerts an anti-proliferation role in CRC by arresting cell cycle in G1/S phase and triggering apoptosis via the Rb-E2F1 signaling.

Key Words:
ATAD2, Colorectal carcinoma, Proliferation, Apoptosis.

Introduction

Colorectal carcinoma (CRC) is a common malignant tumor in the world[1,2], and its mortality and incidence remain high in China[3,4]. Although therapeutic strategies and medical technologies have been largely progressed, the survival of CRC is far away from our expectation because of the high rate of advanced CRC[5]. The pathogenesis and etiology of CRC are complex[6]. Molecular mechanisms underlying CRC progression should be clearly identified for enhancing the clinical outcomes.

ATAD2 (ATPase family, AAA domain containing 2) is a recently detected oncogene[7]. It locates on chromosome 8q24.13, where is often amplified in different types of tumors[8]. ATAD2 is capable of regulating gene expressions or modifying chromatins *via* activating transcription factors[9-11]. ATAD2 is abnormally expressed in tumor profiling and involved in tumor progression, including papillary thyroid carcinoma, gastric cancer, pancreatic cancer, and lung carcinoma[12-15]. The overexpression of ATAD2 is considered to have a relation to poor clinical outcomes of tumors and displays a prognostic potential[16,17].

E2Fs are members of an evolutionarily conserved family of transcription factors and have been extensively studied in tumor progression[18]. E2Fs family contains ten proteins encoded by eight different genes, which are known for their functions in cell cycle regulation. It is reported that E2F and Myc co-regulate cell cycle progression in either normal cells or Rb-deficient cells[19]. Therefore, deficiency of E2F will cause disordered cell cycle progression and thus induce tumorigenesis[20]. ATAD2 can be served as a cofactor with certain transcription factors (such as c-Myc and E2F) to regulate the genes with carcinogenic functions. ATAD2 upregulates proliferation-associated and anti-apoptotic genes in tumors through acting on

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c-Myc and E2F transcription factors. This study aims to clarify the potential biological function of ATAD2 during the progression of CRC and the involvement of the Rb-E2F signaling, and thus provides novel ideas for clinical targeted therapy.

Patients and Methods

Sample Collection

A total of 54 cases of CRC and paracancerous tissues were surgically collected from CRC patients with complete clinical data and follow-up information. Inclusion criteria: none of the patients received preoperative radiochemotherapy, patients with distant metastasis or those that received pre-operative chemo-radiation were excluded from the study. Exclusion criteria: patients complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. The samples were frozen in liquid nitrogen and stored at -80°C. This study was approved by the research Ethics Committee of The First People's Hospital of Yancheng and each patient signed the informed consent.

Cell Culture

The intestinal epithelial cell line (NCM460) and CRC cell lines (DLD1, SW620, HCT15, HCT116 and HCT8) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in DMEM at 37°C with 5% CO_2. 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin were added in culture medium. Cell passage was conducted every 2-3 days.

Plasmid Construction and Cell Transfection

After 48 hours of co-transfecting pLV-ATAD2 shRNA or pLV-control with lentiviral packaging vectors into HEK-293T cells, lentiviruses were harvested for transfection in SW620 and HCT15 cells using Lipofectamine 2000 (Invitrogen, CA, USA). Transfected cells with ATAD2 shRNA or NC for one week were screened by application of 2 μg/mL puromycin. ATAD2 shRNA sequences were 5′-GGTTGTAGCTCCTCCAAAC-3′; and NC sequences were 5′-GTTCTCCGAACGTGTCACGT-3′.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular or tissue RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was used for qRT-PCR. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. ATAD2 primers were as follows: 5'-GGAATCCCAAACCACTGGACA-3' (forward) and 5'-GGTAGCGTCGTCGTAAAGCACA-3' (reverse); β-actin primers were as follows: 5'-CGCAAAGACCTGTATGCCAA-3' (forward) and 5'-CACACAGAGTACTTGCGCTC-3' (reverse).

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 30 min, and centrifuged at 4°C, 14000×g for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure using enhanced chemiluminescence (ECL).

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At day 1, 2, 3 and 4, absorbance value at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in the 6-well plate with 500 cells/well. Medium was replaced once a week in the first week, and twice in the second week. Visible colonies containing more than 50 cells were washed by phosphate-buffered saline (PBS), fixed in methanol for 20 min, and dyed with 0.1% crystal violet for 20 min. Colonies were captured and calculated.

Flow Cytometry

Cells were collected and centrifuged at 1200 rpm for 5 min. After washing in PBS for 2-3 times, the cells were fixed in 75% ethanol at -20°C overnight. On the other day, cells were washed twice and incubated with RNase. They were subjected to incubation with 500 μL of Propidium Iodide

(PI) for 15 min, followed by detecting distribution of cells in each phase of cell cycle. For assessing apoptosis, the cells were subjected to incubation with 10 μg/mL PI and 50 μg/mL Annexin V-FITC in the dark for 15 min, followed by apoptosis detection using flow cytometry (FACScan, BD Biosciences, Detroit, MI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for data analysis. Data were expressed as mean±SD (standard deviation). Statistically significant differences between the groups were estimated by the Student's *t*-test. Correlation between ATAD2 and clinical characteristics of CRC patients was determined by χ^2-test. Kaplan-Meier method was introduced for survival analysis. Univariable and multivariable Cox regression models were generated to illustrate potential risk factors for survival of CRC. $p<0.05$ considered the difference was statistically significant.

Results

Upregulated ATAD2 In CRC

A total of 54 cases of CRC were collected in our hospital. Through qRT-PCR detection, ATAD2 was upregulated in CRC tissues than paracancerous ones (Figure 1A). Kaplan-Meier survival curves revealed a lower survival rate in CRC patients expressing high level of ATAD2 compared with whom expressing a low level (HR=2.2520, $p=0.0389$) (Figure 1B). It is suggested that ATAD2 predicted a poor prognosis of CRC. Subsequently, ATAD2 was detected to be highly expressed in CRC cell lines than that of the intestinal epithelial cell line (Figure 1C). Notably, SW620 and HCT15 cells expressed the relatively high level of ATAD2, and they were selected for the following experiments.

Correlation Between ATAD2 and Clinical Characteristics of CRC

Based on the median level of ATAD2 in CRC tissues, patients were assigned into high level group and low level group for analyzing differences in their clinical characteristics. No significant differences in age, gender, and presence of lymphatic metastasis were identified between groups ($p>0.05$). However, CRC patients expressing high level of ATAD2 presented larger tumor size, worse histological classification and more advanced TNM staging compared with those in low level group ($p<0.05$) (Table I).

Risk Factors for the Overall Survival of CRC

Univariable Cox regression analysis was conducted to assess potential risk factors for the prognosis of CRC. It is shown that ATAD2 level, histological classification, TNM staging, and tumor size were associated with the prognosis of CRC (HR=2.678, 2.208, 2.384 and 2.631, respectively) ($p<0.05$). Furthermore, multivariable Cox regression analysis obtained the similar conclusion that ATAD2 level, histological classification, TNM staging, and tumor size were independent prognostic factors for the prognosis of CRC (HR=2.260, 2.302, 2.335 and 2.492, respectively) ($p<0.05$) (Table II).

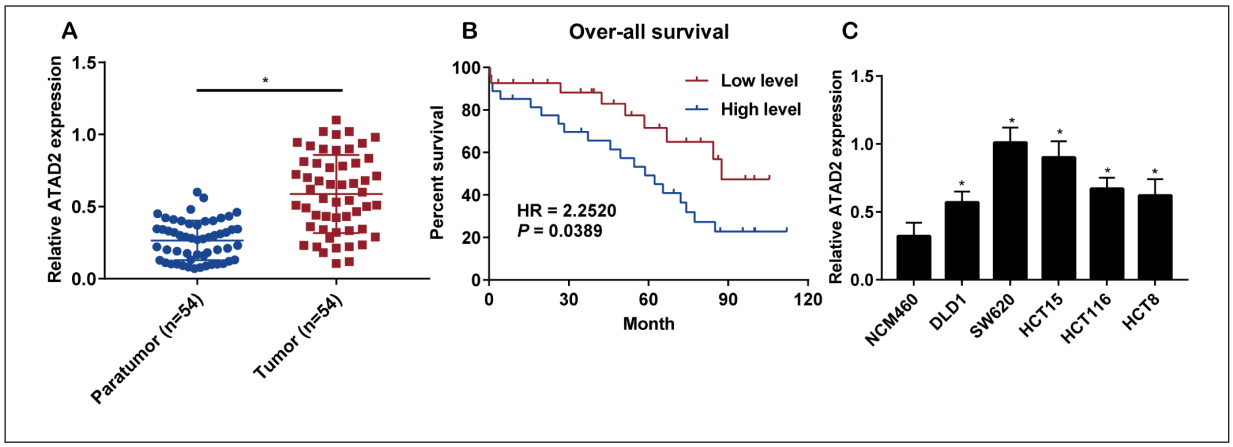

Figure 1. Upregulated ATAD2 in CRC. **A**, ATAD2 level was upregulated in CRC tissues (n=54) than that of paracancerous ones (n=54). **B**, Kaplan-Meier survival curves depicted worse survival in CRC patients with high level of ATAD2. **C**, ATAD2 level was upregulated in CRC cell lines than the intestinal epithelial cell line.

Table I. Correlation between ATAD2 and clinical characteristics of CRC (n=54).

Clinicopathologic features	No. of cases	ATAD2 expression		*p*-value
		Low(n=27)	High(n=27)	
Age (years)				0.414
≤60	27	15	12	
>60	27	12	15	
Gender				0.780
Male	21	11	10	
Female	33	16	17	
Tumor size				0.001*
≤5 cm	26	19	7	
>5 cm	28	8	20	
TNM stage				0.013*
I-II	23	16	7	
III-IV	31	11	20	
Histological classification				0.028*
Low grade	24	16	8	
Medium and high grade	30	11	19	
Lymph node metastasis				0.586
Absent	26	14	12	
Present	28	13	15	

Knockdown of ATAD2 Suppressed In Vitro Proliferative Ability of CRC

Transfection of ATAD2 shRNA in SW620 and HCT15 cells remarkably downregulated ATAD2 (Figure 2A). Subsequently, CCK-8 assay uncovered that the knockdown of ATAD2 reduced viability in SW620 and HCT15 cells (Figure 2B, 2C). In addition, colony number was declined after transfection of ATAD2 shRNA in CRC cells, further verifying the attenuated proliferative ability (Figure 2D, 2E).

Knockdown of ATAD2 Blocked Transition of G1/S Phase In CRC

Flow cytometry was conducted to evaluate changes in cell ratios in different phases of cell cycle progression and apoptosis in CRC cells regulated by ATAD2. The knockdown of ATAD2 elevated cell ratio in G0/G1 phase and declined that in S phase, while cell ratio in G2/M phase was not affected (Figure 3A, 3B). Besides, apoptotic cell rate was markedly increased by the knockdown of ATAD2 in CRC (Figure 3C, 3D).

ATAD2 Regulated Cell Cycle Proteins and Apoptosis-Associated Genes In CRC

The role of ATAD2 in regulating G1/S phase transition and apoptosis in CRC has been identified. Subsequently, regulatory effects of ATAD2 on cell cycle proteins and apoptosis-associated genes were detected by Western blot. Knockdown of ATAD2 downregulated protein levels of Cyclin D1, ppRb, E2F1 and Cyclin E (Figure 4A). No significant difference in protein level of pRb was

Table II. Univariable and multivariable Cox regression analyses on risk factors for prognosis of CRC.

	Univariate analysis			Multivariate analysis		
	p-value	HR	95% CI	*p*-value	HR	95% CI
Age	0.124	1.426	0.274,1.164	-	-	-
Gender	0.212	0.426	0.215,1.216	-	-	-
Tumor size	0.021	2.631	1.318,5.985	0.019	2.492	1.102,3.543
Stage	0.016	2.384	1.169,4.795	0.024	2.335	1.121,3.927
Histological classification	0.027	2.208	1.175,3.849	0.023	2.302	1.063,3.791
Lymph node metastasis	0.324	1.028	0.715,1.487	-	-	-
ATAD2	0.005	2.678	1.221,4.804	0.016	2.260	1.514,3.975

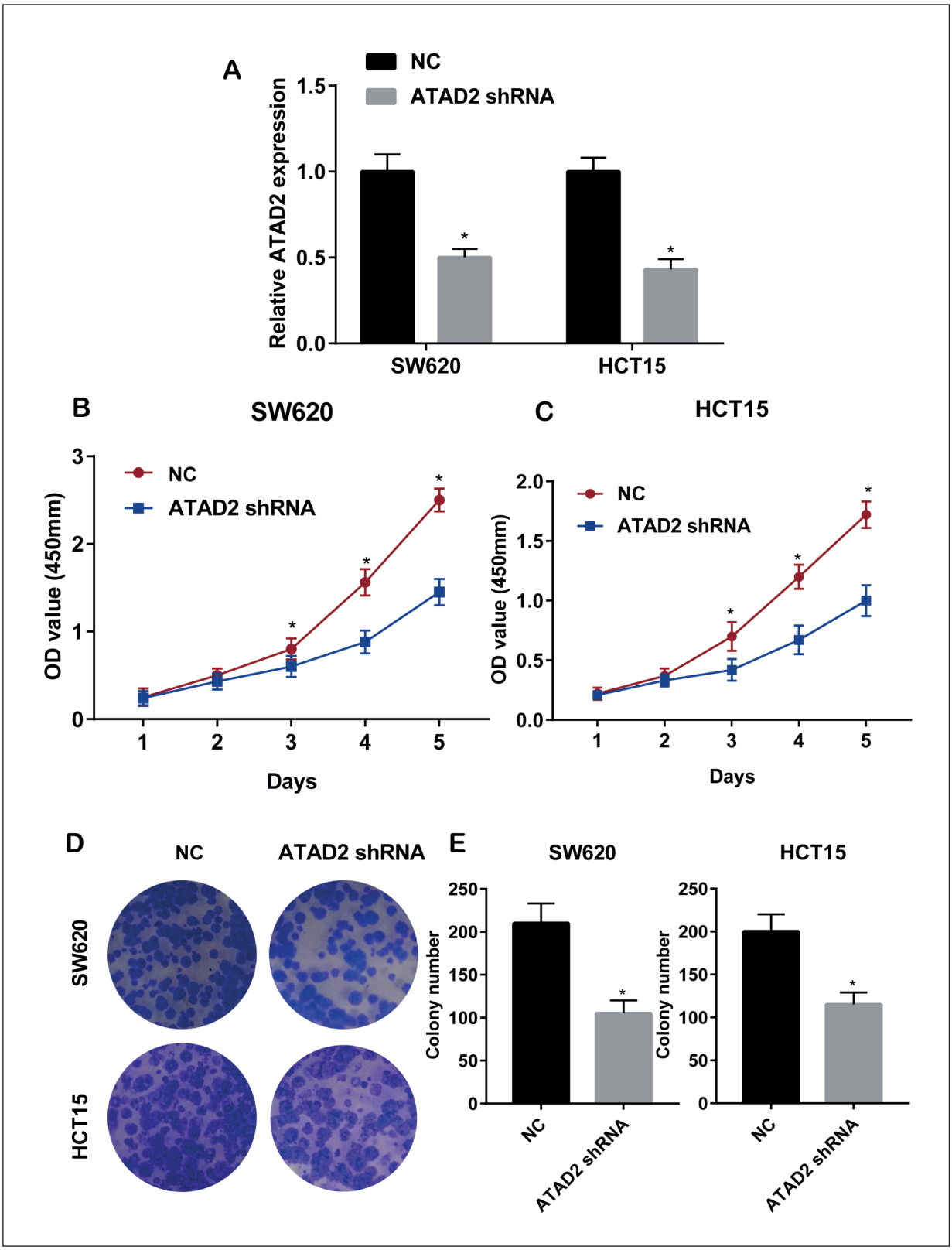

Figure 2. Knockdown of ATAD2 suppressed in vitro proliferative ability of CRC. **A**, Transfection of ATAD2 shRNA significantly downregulated ATAD2 in SW620 and HCT15 cells. **B-C**, Knockdown of ATAD2 declined viability in SW620 (**B**) and HCT15 cells (**C**). **D**, Knockdown of ATAD2 reduced clonality in SW620 and HCT15 cells (magnification: 10×). **E**, Knockdown of ATAD2 reduced colony number in SW620 and HCT15 cells.

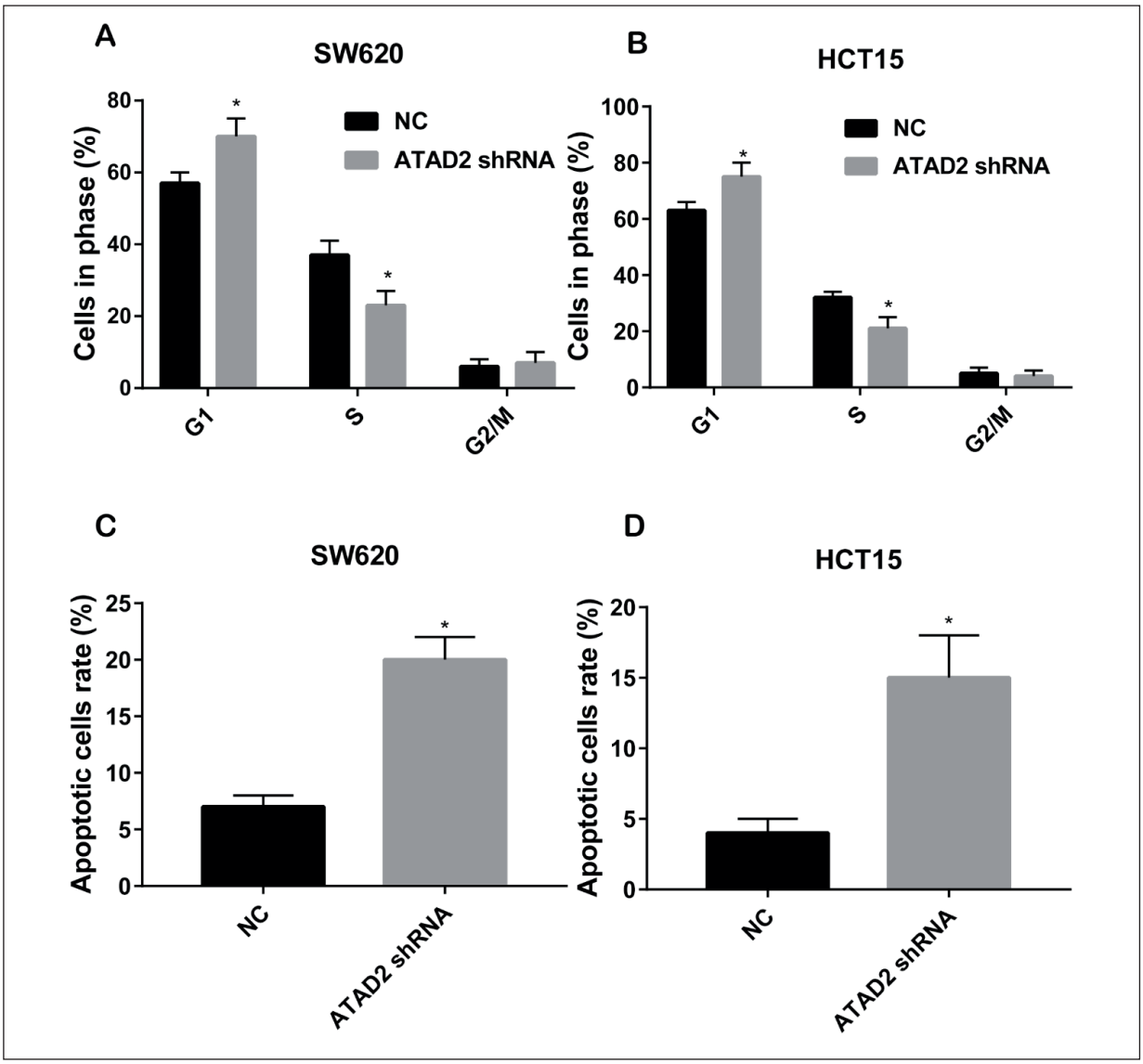

Figure 3. Knockdown of ATAD2 arrested G1/S phase transition in CRC. **A-B**, Knockdown of ATAD2 in SW620 **(A)** and HCT15 cells **(B)** increased cell ratio in G1 phase and decreased that in S phase; **C-D**, Knockdown of ATAD2 increased apoptotic cell rate in SW620 **(C)** and HCT15 cells **(D)**.

observed. In addition, protein level of cleaved Caspase 3 was upregulated by the knockdown of ATAD2 (Figure 4B).

Discussion

Due to the rapid prevalence of CRC in the world, seeking for effective diagnostic and prognostic indicators is urgently required. Previous studies[21-24] have demonstrated the upregulation of ATAD2 in malignant tumors. Consistently, we found that ATAD2 was upregulated in CRC tissues than paracancerous ones. The survival rate of CRC patients with high expression of ATAD2 was lower than that of patients expressing a low level, suggesting that highly expressed ATAD2 was linked to poor prognosis in CRC. In addition, ATAD2 had a close relation to tumor size, histological classification, and TNM staging in CRC. High level of ATAD2 was an independently prognostic factor for CRC. The above findings indicated that ATAD2 may be a promising biomarker for CRC.

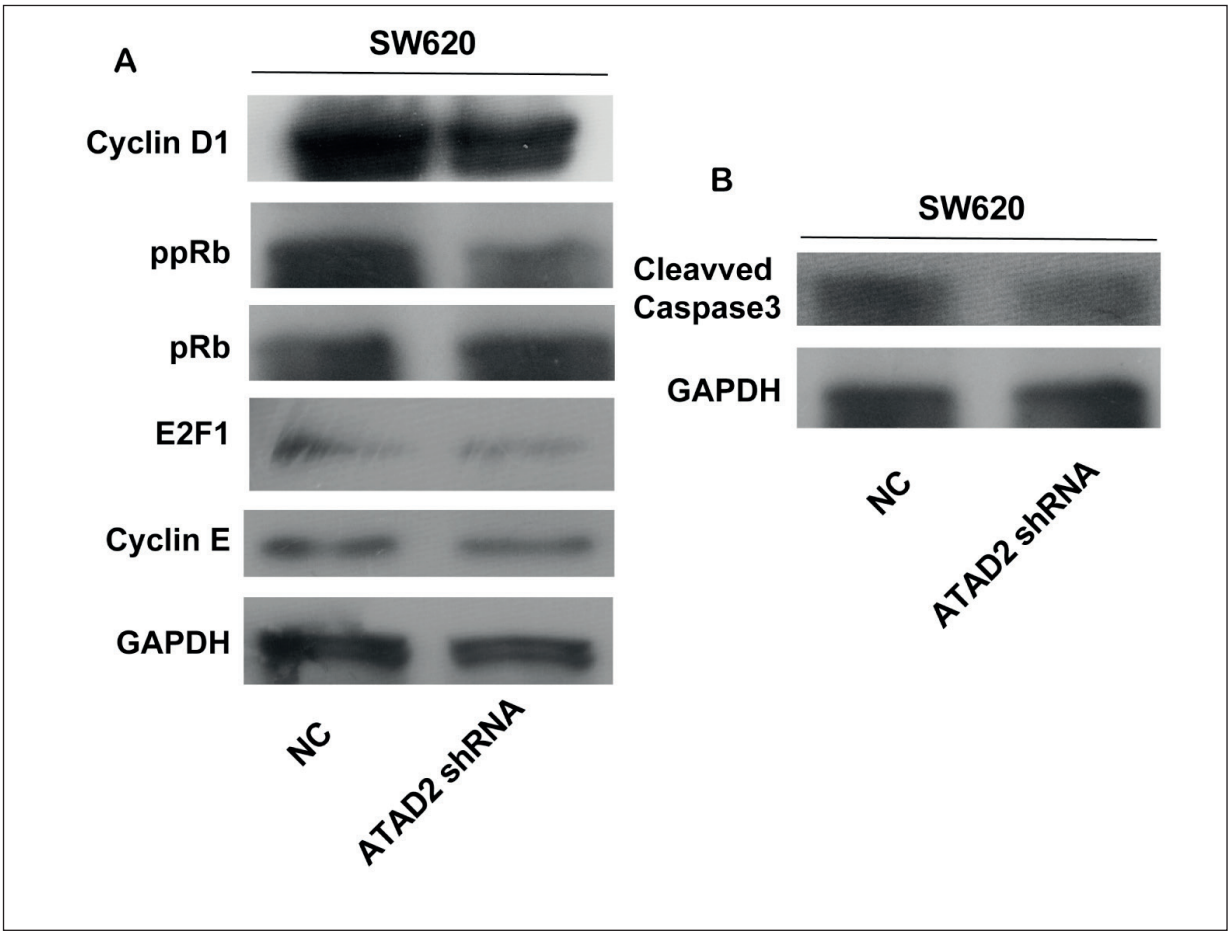

Figure 4. ATAD2 regulated cell cycle proteins and apoptosis-associated genes in CRC. **A**, Knockdown of ATAD2 downregulated protein levels of Cyclin D1, ppRb, E2F1 and Cyclin E in SW620 cells. **B**, Knockdown of ATAD2 upregulated protein level of cleaved Caspase 3 in SW620 cells.

ATAD2 is a vital determinant for tumor proliferation and metastasis[25,26]. The knockdown of ATAD2 in SW620 and HCT15 cells remarkably attenuated viability and clonality. Furthermore, the knockdown of ATAD2 arrested cells in G1/S phase and induced apoptosis in CRC, which were consistent with a previous conclusion leading that the silence of ATAD2 suppresses hepatocellular carcinoma proliferation *via* activating apoptosis signaling mediated by p53 and p38[27].

ATAD2 is a direct target of the pRB/E2F signaling and a co-factor for MYC-regulated Hedgehog pathway[28,29]. Rb protein (pRb) is a tumor-suppressor gene that is responsible for regulating cell cycle progression and tumor process. It is reported that pRb induces terminal differentiation through arresting cell cycle progression from G1 phase to S phase by blocking G1 checkpoint, and regulating tissue-specific gene expressions[30]. Phosphorylation of pRb relies on cell cycle progression, which is mainly regulated by cyclins/cyclin dependent kinases (CDKs)[31]. Rb binds and inhibits E2Fs activators (E2F1, E2F2 and E2F3a), thus triggering cyclin expressions and cell cycle progression into S phase[32]. The function of Rb, including the ability to interact with E2F, is regulated by phosphorylation with the involvement of Cyclin D[33-35]. The role of E2F transcription factors in controlling the transition from G1 phase to S phase has been confirmed[36,37]. Here, protein levels of Cyclin D1, ppRb, E2F1, and Cyclin E were downregulated by transfection of ATAD2 shRNA in SW620 cells. It is well known that Cyclin D1 and Cyclin E are indicated targets of E2F1, which are responsible for inducing transition of G1/S phase[38,39]. Hence, we believed that through

the Rb-E2F1 signaling, ATAD2 blocked transition of G1/S phase in CRC by regulating Cyclin D1 and Cyclin E. Furthermore, the knockdown of ATAD2 induced apoptosis in CRC and upregulated protein level of cleaved Caspase3. Apoptosis signaling, as a result, was also involved in ATAD2-regulated anti-proliferation of CRC. This study elucidated the vital function of ATAD2 in the process of CRC. We suggest that ATAD2 may be a promising prognostic indicator and therapeutic target for CRC, which provides novel references for clinical treatment.

Collectively, the upregulated ATAD2 in CRC induces blockage of G1 phase and apoptosis, thus inhibiting the proliferative ability. Meanwhile, ATAD2 is an independent factor for the overall survival of CRC, and it can be utilized as a candidate for targeted therapy.

Conclusions

These results demonstrated that the knockdown of ATAD2 induces arrestment of G1 phase and apoptosis through the Rb-E2F1 pathway, thereby inhibiting CRC proliferation. ATAD2 may be a promising therapeutic target for CRC, showing a great value in clarifying the occurrence and progress of CRC.

Conflict of Interests

The authors declare that they have no conflict of interests..

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