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***Basic Study***

**Anti-silencing function 1B knockdown suppresses the malignant phenotype of colorectal cancer *via* inactivating the phosphatidylinositol 3-kinase/AKT pathway**

Yu GH *et al.* Decreased ASF1B prevents tumorigenesis of CRC

## **Abstract**

### **BACKGROUND**

Mounting studies have highlighted the pivotal influence of anti-silencing function 1B (ASF1B) on malignancy of cancers.

### **AIM**

To explore the influence and mechanism of ASF1B in colorectal cancer (CRC).

### **METHODS**

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect mRNA expression of ASF1B. Immunohistochemical staining was performed to detect protein expression of ASF1B and Ki67 in tumor tissues. Western blot analysis was used to determine levels of ASF1B and proliferation/epithelial mesenchymal transition (EMT)/stemness-related proteins. Besides, the proliferation of CRC cells was assessed using Cell Counting Kit-8 and 5-Ethynyl-2'-Deoxyuridine assays. The migration and invasion of CRC cells were evaluated using transwell assays. Stemness of CRC cells was tested through sphere formation assay. To construct a xenograft tumor model, HCT116 cells were introduced into mouse flanks *via* subcutaneous injection.

### **RESULTS**

ASF1B expression was markedly increased in CRC tissues and cells, and it was inversely correlated with overall survival of CRC patients and was positively associated with the tumor node metastasis (TNM) stage of CRC patients. Silencing of ASF1B suppressed proliferation, migration, invasion, stemness and EMT of CRC cells as well as tumorigenesis of xenograft mice. Furthermore, protein levels of P-phosphatidylinositol 3-kinase (p-PI3K) and p-AKT were decreased after silencing of ASF1B in CRC cells. The inhibitory effects of ASF1B knockdown on cell proliferation, stemness and EMT were partly abolished by PI3K activator in CRC cells.

## CONCLUSION

Silencing of ASF1B inactivated the PI3K/AKT pathway to suppress CRC malignancy *in vitro*.

**Key Words:** Colorectal cancer; Anti-silencing function 1B; Phosphatidylinositol 3-kinase/AKT; Stemness; Epithelial mesenchymal transition

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**Core Tip:** Anti-silencing function 1B (ASF1B) expression was increased in colorectal cancer (CRC) tissues and cells, and was negatively associated with prognosis of CRC patients. Functionally, ASF1B knockdown repressed malignant behaviors of CRC cells *in vitro* and tumorigenesis *in vivo*, therefore having potential for CRC treatment. Moreover, our findings showed that ASF1B down-regulation suppressed malignant behaviors of CRC cells by inactivating the PI3K/AKT pathway.

## INTRODUCTION

Colorectal cancer (CRC) refers to a malignant gastrointestinal tumor, accounting for 8% of cancer-related deaths in 2018<sup>[1,2]</sup>. According to statistics, it leads to over 1 million deaths every year<sup>[3,4]</sup>. Despite great progress in CRC treatment including surgery, chemotherapy and combined therapy, CRC prognosis is still poor<sup>[5,6]</sup>. Therefore, deeper exploration of CRC pathogenesis and treatment targets is of importance to improve CRC therapy.

For the past few years, molecular targeting treatment emerges as a research hotspot in CRC therapy<sup>[7,8]</sup>. <sup>1</sup>Anti-silencing function 1 (ASF1), a conserved histone H3-H4 chaperone protein, is involved in regulation of many processes such as transcription, DNA damage repair and DNA replication<sup>[11,12]</sup>. Of note, it includes two paralogous

forms: ASF1A histone chaperone and ASF1B<sup>[13]</sup>. ASF1A is primarily implicated in regulation of DNA repair and cellular senescence, while ASF1B acts as a crucial regulator of cellular proliferation and cell cycle progression<sup>[12,14]</sup>. As a subtype of ASF1, up-regulation expression of ASF1B is reported to associate with the poor prognosis of lung adenocarcinoma and breast cancer patients<sup>[15,16]</sup>. More importantly, ASF1B down-regulation is demonstrated to have the ability of anti-tumor in many cancers. For example, ASF1B knockdown suppresses cell proliferation, and promotes cell cycle arrest and apoptosis in cervical cancer<sup>[19]</sup>. ASF1B knockdown impairs proliferation, migration and invasion of lung cancer cells<sup>[20]</sup>. Silencing of ASF1B represses growth of hepatocellular carcinoma (HCC) cells, and induces cell cycle arrest<sup>[21]</sup>. Overall, ASF1B is gaining our attention as an important player in the development of diverse cancers. Meantime, the function and mechanistic understanding of ASF1B have rarely been reported in CRC.

The phosphatidylinositol 3-kinase (PI3K)/Akt pathway is widely found in tumors, and it plays an important role in growth and proliferation of tumor cells<sup>[22]</sup>. Notably, the PI3K/Akt pathway is demonstrated to involve in regulation of CRC progression and development<sup>[23,24]</sup>. For example, pleckstrin homology like domain family A member 2 down-regulation inactivates the PI3K/AKT pathway to inhibit proliferation, invasion and migration of CRC cells<sup>[23]</sup>. Pyrroline-5-carboxylate reductase 2 down-regulation suppresses the PI3K/AKT pathway to prevent proliferation, migration and invasion of CRC cells<sup>[24]</sup>. Inosine 5'-monophosphate dehydrogenase type II knockdown attenuates proliferation, invasion and migration abilities of CRC cells *via* inactivating the PI3K/AKT pathway<sup>[25]</sup>. Especially, ASF1B is revealed to affect malignant behaviors of prostate cancer cells and pancreatic cancer cells *via* regulating the PI3K/AKT pathway<sup>[26,27]</sup>. Thus, we assumed that r ASF1B may regulate the PI3K/AKT pathway to affect malignant progression of CRC. In this study, the expression pattern of ASF1B as well as the role of ASF1B was determined in CRC *in vitro* and *in vivo*. Furthermore, the molecular mechanism of ASF1B-mediated carcinogenesis was explored in CRC cells.

## **MATERIALS AND METHODS**

### ***Tissue samples***

Total 68 pairs of CRC tissues and adjacent normal tissues (3.0 cm away from tumor margin) were collected from CRC patients between June 2019 and January 2021, which were transported by liquid nitrogen to the laboratory and then stored at -80 °C until use. Pathological diagnosis of CRC patients (age range, 23-61 years old; 35 males and 33 females) was conducted by three pathologists based on the eighth edition of the Union for International Cancer Control and the American Joint Committee on Cancer tumor node metastasis (TNM) classification<sup>[28,29]</sup>. The inclusion criteria were as follows: (1) Complete general information (including gender, age, ethnicity, past history and family history); and (2) Patients who underwent bidirectional endoscopy (colonoscopy performed immediately after gastroscopy). The exclusion criteria were as follows: (1) History of gastric cancer, peptic ulcer and other cancers; (2) Received antibiotics, proton pump inhibitors or glucocorticoids in the past month; (3) Patients who underwent chemotherapy, radiation therapy and other treatments for tumors; (4) Previous history of gastrointestinal surgery; (5) Presence of inflammatory bowel disease, Gardner's syndrome (a disease that affects the incidence of CRC) or familial adenoma; and (6) A history of systemic diseases. The current study has got permission from the Ethics Committee of Zhebei Mingzhou Hospital (ZBMZYYLL211028), and all subjects signed informed consents. Besides, CRC patients were divided into ASF1B high or low-expression group based on the median value of ASF1B expression.

### ***Follow-up***

The survival of patients was followed for 90 mo through the records of reexamination or the telephone.

### ***Online website***

The online website GEPIA (<http://gepia.cancer-pku.cn/detail.php>) was used to compare the expression of ASF1B between tumor tissues and normal tissues in CRC.

### *Cell culture*

A human normal colorectal mucosal cell line (FHC) and CRC cell lines (HT29, HCT116, LOVO, SW480 and SW620) were bought from the American Type Culture Collection (ATCC, Manassas, VA, United States). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, United States) containing 10% fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, United States) and 1% penicillin/streptomycin (Gibco), which were incubated at 37 °C under 5% CO<sub>2</sub>. For eliminating mycoplasma contamination, all cells were routinely examined by using MycAway (Yeasen, Shanghai, China).

### *Cell transfection*

Short hairpin RNAs against ASF1B (sh-ASF1B#1, sh-ASF1B#2 and sh-ASF1B#3) and sh-negative control (sh-NC) were bought from RiboBio (Guangzhou, China). Then HCT116 and SW620 cells were seeded in six-well plates to adjust the cell density to  $5 \times 10^5$ /mL. When the cell confluence reached 70%-80%, above plasmids (final concentrations, 50 nM) were transiently transfected into cells through Lipofectamine 3000 (Invitrogen) for 48 h. To further explore the role of PI3K/Akt pathway in CRC cells, transfected HCT116 cells were treated with PI3K activator (740 Y-P, 50 ug/mL, MedChemexpress, Shanghai, China) for 90 min<sup>[30]</sup>.

### *Quantitative real-time polymerase chain reaction*

Total RNAs of CRC tissues were isolated using TRIzol reagent (Invitrogen), and then were used for synthesis of complementary DNAs (cDNAs) according to instructions of a PrimeScript RT reagent kit (Takara, Dalian, China). Next, cDNAs and the SYBR Green polymerase chain reaction (PCR) kit (Takara) were used to perform quantitative real-time PCR (qRT-PCR) on a LightCycler 480 Real-Time PCR system (Roche, Shanghai, China). Reaction procedures for PCR were displayed as follows: 5 min at 94 °C, 38 cycles of 25 s at 94 °C, 1 min at 60 °C and 2 min at 72 °C. Primers bought from Sangon

(Shanghai, China) were listed as follows: ASF1B-F, GATCAGCTTCGAGTGCAGTG; ASF1B-R, TGGTAGGTGCAGGTGATGAG; GAPDH-F, CCATCTTCCAGGAGCGAGAT; GAPDH-R, TGCTGATGATCTTGAGGCTG. Relative mRNA expression of ASF1B, which was normalized to GAPDH, was calculated through the  $2^{-\Delta\Delta Ct}$  method.

#### ***Cell Counting Kit-8 assay***

Proliferation of HCT116 and SW620 cells was assessed through a Cell Counting Kit-8 (CCK-8) kit (Beyotime, Shanghai, China). Briefly, transfected cells ( $5 \times 10^3$  cells/well) were plated into 96-well plates, followed by incubation for 0 h, 24 h, 48 h, 72 h and 96 h. After that, CCK-8 solution (10  $\mu$ L) was added to each well to incubate at 37 °C for 2 h. Finally, the optical density at 450 nm was measured using a microplate reader (MG LABTECH, Durham, NC, United States).

#### ***5-Ethynyl-2'-Deoxyuridine assay***

Proliferation of HCT116 and SW620 cells was assessed using 5-Ethynyl-2'-Deoxyuridine (EDU) proliferation assay (RiboBio). In detail, HCT116 and SW620 cells ( $1 \times 10^5$  cells/well) were plated to 96-well plates. Then these cells were exposed to 50  $\mu$ M EDU at 37 °C for 2 h, and fixed with 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 20 min. Afterwards, EDU solution was added to culture, followed by staining of nuclei *via* Hoechst. EDU-positive cells were observed using a fluorescent microscope.

#### ***Transwell assay***

The invasion of HCT116 and SW620 cells was assessed using the transwell chamber (8.0  $\mu$ m pore size; Millipore, Billerica, MA, United States) coated with Matrigel. In brief, cells (100000 cells/well) re-suspended in FBS-free medium were put onto the upper compartment. Subsequently, the upper compartment was filled with 600  $\mu$ L culture medium with 10% FBS. After chambers were incubated for 24 h, cells in the lower



surface were fixed with 4% paraformaldehyde and were dyed with 0.1% crystal violet. Invasive cells were counted under an optical microscope. In contrast, cell migration was assessed through the upper chamber without Matrigel, and the procedures were the same as invasion evaluation.

#### *Sphere formation assay*

Stemness of HCT116 and SW620 cells was evaluated through sphere formation assay. Briefly, cells (250 cells/well) were put into 24-well plates with ultra-low attachment, which were cultured in RPMI 1640 medium containing 10 ng/mL fibroblast growth factor, 10 µg/mL insulin, 10 ng/mL epidermal growth factor and 2% B-27. After 10 d of incubation, representative fields were photographed using a Nikon microscope.

#### *Western blot analysis*

Protein extraction of CRC tissues and cells was performed using RIPA lysis buffer (Beyotime). Then lysates were centrifuged (12000 r/min) for 10 min at 4 °C, and 5 × loading buffer was added to the supernatant to denature at 100 °C for 5 min. Determination of protein concentration was performed using a BCA Protein Assay Kit (Beyotime). Subsequently, the sodium dodecyl sulphate-polyacrylamide gel with 5% stacking gel and 10% separation gel was prepared, and these protein samples (10 µL) were separated through electrophoresis in 1 × buffer [500 mL ddH<sub>2</sub>O, 9.4 g glycine, 1.51 g Tris-base and 0.5 g sodium dodecyl sulfate (SDS)] at 80 V for 30 min and at 120 V for 1 h. Next, proteins were subjected to 10% SDS polyacrylamide gel electrophoresis, after which proteins were transferred to polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany). After being blocked with 5% skim milk, primary antibodies (Abcam, Cambridge, CA, United States) including anti-ASF1B (1:1000, ab276071), anti-E-cadherin (1:10000, ab40772), anti-N-cadherin (1:1000, ab245117), anti-SOX2 (1:1000, ab92494), anti-OCT4 (1:10000, ab200834), anti-p-PI3K (1:500, ab278545), anti-PI3K (1:1000, ab32089), anti-p-AKT (1:500, ab38449), anti-AKT (1:500, ab8805) and anti-β-actin (1:200, ab115777) were added to immerse membranes at 4 °C overnight. Then the

secondary antibody (1:2000, ab6721) was added. Finally, the ECL chemiluminescent system was used to examine protein blots and Image J software (NIH, United States) was used to analyze the intensity of protein blots.

### ***Immunohistochemistry staining***

Tumor tissues of mice fixed with 4% paraformaldehyde were embedded using paraffin and cut into sections (4  $\mu$ m), and were dried at 62 °C for 1 h. Next, these sections were deparaffinized using xylene for 20 min, and were soaked in 100%, 95%, 90%, 80% and 70% ethyl alcohol for 5 min. Then antigen repair was performed at 120 °C in citrate buffer (pH 6.0), and the tissue sections were cooled at room temperature. After being washed *via* phosphate buffered saline (PBS), incubated in H<sub>2</sub>O<sub>2</sub> and washed *via* PBS again, sections were blocked using bovine serum albumin (5%) for 1 h. Subsequently, primary antibodies including anti-ASF1B (1:200, ab235358, Abcam) and anti-Ki67 (1:200, ab16667, Abcam) were added to incubate overnight. And the secondary antibody (1:500, ab6112, Abcam) was added, followed by addition of 3,3'-diaminobenzidine substrate solution. Finally, through hematoxylin was used to stain these samples, and staining areas were observed through a microscope.

### ***Xenograft assay***

Animal experiments have got permission from the Institutional Animal Care and Use Committee of Beijing Viewsolid Biotechnology Co. LTD (VS212601454), which complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as ARRIVE guidelines. Female BALB/C nude mice (5-wk old), were purchased from Chinese Academy of Sciences (Shanghai, China), and were kept at 25 °C under a condition with a 12 h light/dark cycle and 50% humidity.

To establish a xenograft mouse model,  $5 \times 10^6$  HCT116 cells transfected with sh-NC or sh-ASF1B#1 were introduced into the right flank of mice *via* subcutaneous injection<sup>[31]</sup>. Mice were randomly divided into two groups ( $n = 5/\text{group}$ ): The sh-NC group and the sh-ASF1B#1 group. The tumor size was gauged every week after

injection. Tumor volume was calculated through the formula  $V = (\text{shortest diameter})^2 \times (\text{longest diameter}) \times 0.5$ . On the 35<sup>th</sup> d after the injection, mice were anesthetized through intraperitoneally injecting sodium pentobarbital (50 mg/kg) and then euthanized through cervical dislocation. Finally, tumors were collected for further analysis.

### *Statistical analysis*

Data from three separate experiments were presented as mean  $\pm$  SD. GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA) was used to perform statistical analysis. Student's *t*-test was performed to analyze differences between two groups. Survival curves were plotted through the Kaplan-Meier method and differences between survival curves were assessed using the log-rank test. Clinicopathological characteristics between patients who demonstrated high ASF1B expression *vs* those with low expression were compared using the  $\chi^2$  test. Any difference with  $P < 0.05$  was considered as statistical significance.

## **RESULTS**

**High expression of ASF1B is observed in CRC tissues and is associated with adverse clinicopathological CRC characteristics**

We firstly analyzed ASF1B expression in TCGA database, and observed up-regulation of ASF1B expression in tissues of colon adenocarcinoma and rectum adenocarcinoma in contrast to corresponding noncancerous tissues (Figure 1A,  $P < 0.05$ ). Then we validated mRNA expression of ASF1B using qRT-PCR. As expected, the mRNA expression of ASF1B was increased in CRC tissues relative to that in normal tissues (Figure 1B,  $P < 0.01$ ). Moreover, the protein expression of ASF1B was detected via immunohistochemistry staining and western blot, and higher ASF1B expression was found in tumor tissues than normal tissues (Figures 1C and 1D,  $P < 0.05$ ). We further investigated the clinical significance of ASF1B in CRC and we found that ASF1B expression was significantly associated with TNM stage, lymph node metastasis and

distant metastasis (Table 1). The expression of ASF1B was markedly higher in CRC patients at TNM stage III/IV than CRC patients at TNM stage I/II (Figure 1E,  $P < 0.01$ ). ASF1B up-regulation resulted in poor survival data of CRC patients (Figure 1F,  $P < 0.01$ ).

#### *Silencing of ASF1B impairs proliferation of CRC cells*

Then, the protein expression of ASF1B was detected by western blot in a normal colorectal mucosal cell line (FHC) and CRC cell lines. Examination by western blot showed that ASF1B expression was increased in HT29, HCT116, LOVO, SW480 and SW620 cells compared to FHC cells ( $P < 0.001$ ) and especially in SW620 and HCT116 cells. Therefore, the impact of ASF1B on CRC cell proliferation was investigated through loss of function assays in HCT116 and SW620 cell lines.

Next, the impact of ASF1B on proliferation of CRC cells was probed through loss-of-function assays. Firstly, ASF1B was silenced by transfection of sh-ASF1B#1 and sh-ASF1B#2 in HCT116 and SW620 cells (Figure 2B,  $P < 0.01$ ), and sh-ASF1B#1 and sh-ASF1B#2 were utilized for subsequent function experiments. Then the influence of ASF1B on proliferation of CRC cells was determined via CCK-8 and EDU assays. It was found that the proliferation ability of HCT116 and SW620 cells was attenuated by ASF1B knockdown (Figures 2C and 2D,  $P < 0.05$ ). At the same time, we found that protein levels of proliferation markers (Ki67 and PCNA) were decreased after ASF1B knockdown in HCT116 and SW620 cells (Figure 2E,  $P < 0.01$ ).

#### *ASF1B down-regulation represses migration, invasion, stemness and epithelial mesenchymal transition of CRC cells*

When we explored influences of ASF1B on cell migration and invasion, suppression of migration and invasion caused by ASF1B knockdown was found in HCT116 cells and SW620 cells (Figures 3A and 3B,  $P < 0.001$ ). In addition, we detected influences of ASF1B on cell stemness and epithelial mesenchymal transition (EMT). It was demonstrated that ASF1B down-regulation led to an evident decrease in sphere

formation of HCT116 and SW620 cells (Figure 3C), indicating that ASF1B down-regulation suppressed cell stemness. Meantime, we examined levels of EMT-related proteins (E-cadherin and N-cadherin) and stemness marker proteins (SOX2 and OCT) using western blot. As expected, <sup>13</sup>we observed the increased level of E-cadherin and the decreased level of N-cadherin as well as decreased levels of SOX2 and OCT after ASF1B down-regulation in HCT116 and SW620 cells (Figure 3D,  $P < 0.05$ ).

#### ***PI3K activator reverses inhibitory impacts of ASF1B down-regulation on proliferation, stemness and EMT of CRC cells***

<sup>14</sup>In order to delve into the underlying mechanism involved in the regulatory impact of ASF1B on CRC malignancy, subsequent experiments were conducted. It was demonstrated that p-PI3K and p-AKT levels were reduced in response to ASF1B knockdown in HCT116 and SW620 cells (Figure 4A,  $P < 0.01$ ). Meanwhile, reduction of p-PI3K and p-AKT levels caused by ASF1B knockdown was reversed by addition of PI3K activator (740 Y-P) in HCT116 cells (Figure 4B,  $P < 0.05$ ). More importantly, the inhibitory effect of ASF1B knockdown on proliferation was partly abolished by addition of PI3K activator in HCT116 cells (Figure 4C). The inhibitory effects of ASF1B knockdown on protein levels of N-cadherin, SOX2 and OCT <sup>4</sup>as well as the promoting effect of ASF1B knockdown on the level of E-cadherin were partly reversed by addition of PI3K activator in HCT116 cells (Figure 4D,  $P < 0.05$ ). These results implied that ASF1B down-regulation made impact on CRC malignancy *via* regulating the PI3K/AKT pathway.

#### ***Silencing of ASF1B represses CRC tumorigenesis in mice***

After detecting the function of ASF1B in CRC *in vitro*, we next verified its function *in vivo*. As illustrated in Figures 5A-C, silencing of ASF1B inhibited tumor growth, and reduced tumor volume and weight in xenograft mice ( $P < 0.05$ ). Likewise, protein levels of Ki67 and ASF1B were reduced after ASF1B knockdown in tumor tissues of mice (Figure 5D).

## DISCUSSION

Over the past years, the mortality rate of CRC patients is increasing and limited prognosis poses a severe threat to CRC patients<sup>[32-34]</sup>. Thus, developing new effective therapies for CRC patients is imperative. Emerging evidence has uncovered that ASF1B is highly expressed and shows potential clinical value for prognosis of cancer patients<sup>[15,21]</sup>. For instance, ASF1B shows high expression in HCC tissues, and ASF1B up-regulation is correlated with poor survival of HCC patients<sup>[21]</sup>. ASF1B expression is increased in tissues of lung adenocarcinoma, and ASF1B up-regulation is correlated with worse overall survival of lung adenocarcinoma patients<sup>[15]</sup>. Here, we also found up-regulation of ASF1B expression in CRC tissues and cells, and our outcome was consistent with prior reports<sup>[16,35]</sup>. Meantime, we discovered that ASF1B expression was significantly associated with TNM stage, lymph node metastasis and distant metastasis. At the same time, ASF1B expression was inversely relevant to survival data of CRC patients, and was markedly higher in CRC patients of advanced TNM stages than patients of earlier stages. These results suggested that ASF1B may be a useful marker for prognosis and diagnosis assessment of CRC patients.

In previous studies, ASF1B is reported to be a key regulator in cancer progression<sup>[19,20]</sup>. For instance, ASF1B knockdown prevents cervical cancer cells from proliferating, migrating and invading, and suppresses tumor growth in mice<sup>[19]</sup>. ASF1B knockdown inhibits migration, invasion and EMT of lung cancer cells, and retards tumor growth of xenograft mice as well as expression of Ki67<sup>[20]</sup>. Similarly, our outcomes *in vitro* displayed that ASF1B down-regulation distinctly attenuated proliferation, migration and invasion abilities of HCT116 and SW620 cells, and inhibited EMT and stemness of HCT116 and SW620 cells. As an important cancer hallmark in metastases and the “cadherin switch”, EMT is uncovered to initiate CRC metastasis from the primary tumor to distant sites, especially to liver and lymph nodes<sup>[36,37]</sup>. It is reported that loss of E-cadherin can cause metastatic dissemination and activation of EMT transcription factors in cancer cells<sup>[38]</sup>. Many invasive and metastatic cancers are



associated with high expression of E-cadherin, notably in prostate cancer<sup>[39]</sup>, ovarian cancer<sup>[40]</sup>, and glioblastoma<sup>[41]</sup>, suggesting that E-cadherin facilitates metastasis in several tumors instead of inhibiting tumor progression. In addition, N-cadherin is reported to act as an indicator of ongoing EMT and N-cadherin down-regulation can cause metastatic dissemination<sup>[42]</sup>. In our study, we found that the silencing of ASF1B results in a reverse cadherin switch phenomenon, indicating a potentially important role of ASF1B silencing in reducing the metastatic potential of CRC cells *in vitro*. At the same time, our results *in vivo* indicated that ASF1B down-regulation suppressed tumor growth of xenograft mice as well as Ki67 expression. These findings provided evidence of the anti-tumor role of ASF1B knockdown in CRC, and shed light on exploring promising therapeutic agents for CRC treatment.

Considering the important function of ASF1B in CRC, we attempted to explore how ASF1B affected CRC progression and development. It is noteworthy that ASF1B is proved to affect progression and development of cancers *via* regulating the PI3K/AKT pathway<sup>[26,27]</sup>. For instance, suppression of the PI3K/AKT pathway is pertinent to the influence of ASF1B knockdown on prostate cancer<sup>[26]</sup>. Silencing of ASF1B impairs cell proliferation by inactivating the PI3K/AKT pathway in pancreatic cancer<sup>[27]</sup>. In this study, we also investigated whether the PI3K/AKT pathway participated in ASF1B knockdown-mediated tumor inhibition in CRC cells. It was found that protein levels of p-PI3K and p-AKT were decreased due to ASF1B knockdown in HCT116 and SW620 cells. Concurrently, suppressive influences of ASF1B knockdown on proliferation, stemness and EMT of HCT116 cells were reversed by PI3K activator. Combining above findings, we concluded that ASF1B knockdown repressed malignant behaviors of CRC cells through inactivation of the PI3K/AKT pathway.

However, there were three limitations in our study. Firstly, we only explored the role of ASF1B knockdown in CRC, and the overexpression experiment of ASF1B should be performed to further validate the influence of ASF1B on CRC. Secondly, only *in vitro* studies have been performed and therefore *in vivo* studies are recommended to strengthen the study's hypothesis. Thirdly, we failed to perform a-priori sample

calculation, and we determined the sample size through literature search. Overall, the novelty of this study included two aspects. Firstly, we demonstrated the role of ASF1B in CRC *in vitro* and *in vivo* for the first time. Secondly, we uncovered a new mechanism by which ASF1B made impact on CRC cells.

## **CONCLUSION**

In summary, ASF1B expression was increased in CRC tissues and cells, and was negatively associated with prognosis of CRC patients. Functionally, ASF1B knockdown repressed malignant behaviors of CRC cells *in vitro* and tumorigenesis *in vivo*, therefore having potential for CRC treatment. Moreover, our findings revealed that ASF1B down-regulation repressed impaired behaviors of CRC cells by inactivating the PI3K/AKT pathway. Our study provides new insights into the functional importance of ASF1B in CRC, and indicates that ASF1B may be a promising prognostic marker and a target for the management of CRC.

ASF1B may be a potential diagnostic and prognostic biomarker for improving CRC patients' outcome. More importantly, ASF1B may be a novel target for the treatment of CRC, showing promising prospects in clinical practice. In our future studies, we plan to perform *in vivo* studies to validate the role of ASF1B in CRC. Moreover, we expect more work in investigation of the other mechanisms of ASF1B in cancers.

## **ARTICLE HIGHLIGHTS**

### ***Research background***

Colorectal cancer (CRC) is identified as a malignant gastrointestinal tumor, with high prevalence and mortality. Abundant studies have proved the important role of anti-silencing function 1B (ASF1B) in cancers, but little is known about ASF1B in CRC.

### ***Research motivation***

In order to identify the prognosis biomarker and treatment target for CRC.



### ***Research objectives***

To probe the role and mechanism of ASF1B in CRC.

### ***Research <sup>7</sup>methods***

The mRNA expression of ASF1B was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The clinical value of ASF1B for diagnosis and prognosis of CRC was assessed. The function of ASF1B was evaluated using *in vitro* assays and *in vivo* tumor formation experiments. The molecular mechanism of ASF1B on the phosphatidylinositol 3-kinase (PI3K)/AKT pathway was explored *via* addition of PI3K activator.

### ***Research results***

The expression level of ASF1B was evidently increased in CRC tissues and cells, which was inversely associated with survival time of CRC patients and positively associated with tumor node metastasis stage of CRC patients. Biological functional analyses indicated that ASF1B knockdown may suppress the malignancy of CRC cells *via* regulating the PI3K/AKT pathway.

### ***Research conclusions***

ASF1B is highly expressed in CRC tissues and cells, showing potential as a diagnostic and prognostic biomarker for CRC. Silencing of ASF1B inactivated the PI3K/AKT pathway to inhibit CRC malignancy *in vitro*.

### ***Research perspectives***

Other mechanisms of ASF1B in CRC may be investigated in the future, and its application in anti-tumor therapy will be extended.

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