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Original Article

WTAP knockdown inhibits cell migration through regulating SNAIL1 expression in colorectal cancer

Jingjing Han[1], Jiankun Zhang[2], Huilong Shi[3], Xiaowen Wen[3]

[1]Department of Gastrointestinal Surgery, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan 250117, Shandong, China; [2]Department of General Surgery, Zhaoyuan People's Hospital, Yantai 265406, Shandong, China; [3]Department of Colorectal Surgery, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, Jinan 250117, Shandong, China

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Abstract: Objective: N6-methyladenosine (m6A) modification is the most prevalent mRNA modification in carcinogenesis and it plays a crucial role. WTAP, an m6A RNA methyltransferase, is functionally significant in various cancers; however, the specific role and functional mechanism in colorectal cancer (CRC) remain poorly understood. Method: In this study, we utilized Gene Expression Profiling Interactive Analysis (GEPIA) to compare WTAP expression in CRC and normal tissues. Functional assays including colony formation assay and transwell assay were conducted to assess the impact of WTAP on cell viability and migration. RNA dot blot and MeRIP-PCR assays were used to investigate WTAP's role in m6A modification. Results: WTAP expression was elevated in CRC tissues. Colony formation and transwell assays showed that WTAP promoted proliferation and migration of CRC cells in vitro. Mechanistically, MeRIP-PCR analysis demonstrated that WTAP knockdown inhibited SNAI1 expression by reducing m6A modification of SNAI1 in CRC cells. Supporting this, analysis of data from GEPIA and cBioPortal revealed a positive correlation between WTAP and SNAI1 expression. Conclusion: WTAP may act as an oncogene in CRC by regulating SNAI1 expression.

Keywords: WTAP, CDH1, SNAIL1, colorectal cancer

Introduction

Colorectal cancer (CRC) is a prevalent malignancy and one of the leading causes of cancer-related death worldwide [1]. Although advancements in treatment strategies have improved the survival rate of CRC patients, recurrence and metastasis remain high. Distant organ metastasis significantly contributes to CRC lethality. Limited diagnostic options pose substantial challenges to effective CRC treatment. Therefore, it is urgent to explore novel molecular markers that regulate CRC progression for better prognostic prediction.

N6-methyladenosine (m6A) is an epigenetic modification that plays crucial roles in physiological and pathological processes, particularly in tumor initiation and progression [2-4]. m6A modification is a dynamic process, involving both methylation and demethylation, and influences RNA fate by regulating RNA stability and translation. This modification is mediated by a series of regulators, including "writers", "erasers", and "readers". m6A RNA methylation is installed by a protein complex known as "writers", including METTL3, METTL14, WTAP, RBM15, VIRMA, and ZC3H13 [5]. METTL3 possesses catalytic activity, while other proteins act as accessory factors to METTL3. Conversely, demethylases ("erasers"), such as FTO and ALKBH5, remove m6A modifications [6]. Additionally, m6A sites on RNAs are recognized by "readers", especially the YT521-B homology (YTH) domain family, which includes YTHDF1, YTHDF2, YTHDF3, YTHDC1, and YTHDC2 [7]. Dysregulation of m6A modification has been linked to tumorigenesis in cancers such as acute myeloid leukemia (AML), lung adenocarcinoma (LUAD), and CRC.

Wilms tumor 1-associated protein (WTAP) facilitates the localization of METTL3/METTL14 heterodimer in the nucleus, enabling its catalytic

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activity [8]. Extensive evidence links WTAP to the progression of various cancers. In AML, WTAP acts as an oncogene, promoting abnormal proliferation and inhibiting differentiation [9, 10]. WTAP-mediated m6A modification facilitates the progression of hepatocellular carcinoma (HCC) by regulating the HuR-EST-p21/p27 axis [11]. WTAP is expressed at low levels in LUAD and is associated with the immune system in LUAD [12, 13]. Additionally, WTAP has been implicated in CRC progression [14, 15], although its function in this context is not fully elucidated. This study, therefore, aims to investigate the relationship between WTAP and SNAI1 in CRC, hoping to provide more insights into the progression of colorectal cancer.

Material and methods

Colorectal cancer tissue specimens

Fourteen specimens from CRC patients, including tumor tissues and their adjacent normal tissues, were collected from Shandong Cancer Hospital and Institute. Patients were included if they had a confirmed colorectal cancer diagnosis and were excluded if they had taken prescription or other medications that could affect study outcomes. The tissues were promptly frozen at -80°C upon collection. Written informed consent was obtained from all patients, and the study was approved by the Ethics Committee of Shandong Cancer Hospital and Institute.

Cell culture and reagents

The human colorectal carcinoma cell lines (HCT-116, SW620, SW480, and HT-29) were purchased from Keygen Biotech (Nanjing, Jiangsu, China). All the cells were cultured in DMEM medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, MA, USA) and 1% Penicillin-Streptomycin (KeyGEN BioTECH, Jiangsu, China).

Cell transfection

HCT-116 or SW680 cells (1×10^5) were seeded in 6-well tissue culture plates. The siRNAs targeting *WTAP* and Lipofectamine RNAiMAX reagent (Invitrogen, Thermo Fisher Scientific, CA, USA) were separately diluted in Opti-MEM medium (Gibco, Carlsbad, CA) and then mixed. After transfection for 6 h, the medium were replaced with fresh complete medium. Cells were harvested 48 hours post-transfection for further analysis.

Two siRNAs used in this study were synthetized by RiboBio (Guangzhou, Guangdong, China). The sequences are as follows: 5'-AAUGAAAGGUGAACUGGAA-3' (siWTAP-1); 5'-GUACACAGAUCUUAACUCU-3' (siWTAP-2). siRNAs transfections were performed using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Crystal violet reagent was obtained from Sigma-Aldrich.

RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was extracted using the SPARKeasy kit (SparkJade, Shandong, China) according to the manufacturer's instructions, and the RNA was used for cDNA synthesis with the SPARKscript 1st Strand cDNA Synthesis Kit (SparkJade, Shandong, China). qRT-PCR was performed on a Roche 480 instrument using the AceQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The primer sequences used were as follows: 5'-TGTGGGCATCAATGGATTTGG-3' (GAPDH forward), 5'-ACACCATGTATTCCGGGTCAAT-3' (GAPDH reverse), 5'-TTGTAATGCGACTAGCAACCAA-3' (WTAP forward), and 5'-GCTGGGTCTACCATTGTTGATCT-3' (WTAP reverse).

RNA m6A dot blot assay and quantification

Total RNA was extracted. The m6A dot blot assay was performed following a published protocol [6]. Briefly, RNA samples were loaded to the PVDF membrane (IPVH0010, Milipore) and crosslinked using an ultraviolet crosslinker. Then, the membrane was blocked and incubated with anti-m6A antibody (1:1000 dilution, ab208577, Abcam), followed by incubation with a secondary antibody at room temperature for 2 hours. Detection was performed using ECL Detection Reagent (PK10002, Proteintech), and the signal from each dot was captured by Minichem™ Chemiluminescence Imaging System (Sagecreation, Beijing, China) in all experiments.

MeRIP-RT-qPCR

The MeRIP assay was performed according to the protocol of RNA Immunoprecipitation Kit

(GS-ET-006, Cloudseq, Shanghai, China). Briefly, cells cultured in 15 cm dishes were washed with PBS and collected in RIP lysis buffer. Both m6A antibody and mouse IgG were incubated with magnetic beads for 1 h at room temperature and then incubated with cell lysate at 2 h at room temperature. After two washes, the beads were eluted and the input and immunoprecipitated RNAs were recovered, extracted and subjected to qRT-PCR analysis. The primers for qRT-PCR are listed as following: Forward 5'-GATTCCTGAGCTGGCCTGTCT-3'; Reverse 5'-GGTGGCCTGAGGGTTCCTTGT-3'.

Colony formation assay

HCT-116 and SW620 cells were seeded at a density of 500 cells per well in 6-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 2 weeks. The cells were fixed with 4% paraformaldehyde (solarbio, Beijing, China) for 30 minutes at 4°C and washed 3 times with PBS. Subsequently, the cells were stained with 0.1% crystal violet for 30 minutes, followed by imaging and quantification.

Western blotting

Cultured cells were lysed in RIPA buffer (P0013B), and cell lysates were centrifuged to collect supernatants. Protein concentrations were measured using the BCA Protein Quantification Kit (E112, Vazyme, China). Fifty micrograms of protein was loaded per sample, separated by SDS-PAGE, and then incubated overnight at 4°C with respective primary antibodies. Subsequently, membranes were incubated with secondary antibodies. Protein signals were detected using Western Bright ECL (PK10003, Proteintech) and a chemiluminescence analyzer.

The following primary antibodies were used: anti-GAPDH (60004, proteintech), anti-WTAP (60188, proteintech), anti-CDH1 (20874, proteintech), anti-CDH2 (22018, proteintech) and anti-SNAIL1 (13099, proteintech).

Trans-well assay

For migration assay, 2×10^5 cells suspended in 200 µl of serum-free medium were placed in the upper chambers (8 µm pore size, BD biosciences, China), and 500 µl serum-containing (15%) medium was added into the lower chambers. The cells were cultured in 5% CO_2 at 37°C for 2 days. Cells that migrated to the bottom chambers were fixed with 4% paraformaldehyde, stained by crystal violet and photographed.

Statistical analysis

WTAP expression and overall survival analyses were conducted using the GEPIA database, and correlation analysis between WTAP and SNAIL1 was performed using data from the cBioPortal dataset [16]. All experiments were performed in triplicate, with experimental data were presented as mean ± standard error. Statistical analyses were conducted using SPSS 21. Student's t-test or one-way ANOVA followed by Tukey test were performed for data comparison. Means were compared using two-way analysis of variance where applicable. Statistical significance was defined as a *P*-value less than 0.05.

Results

WTAP expression was elevated in colorectal cancer

While some evidence suggests that WTAP correlates with CRC progression [14, 15, 17], few studies have elucidated the mechanism of WTAP in the proliferation and migration of CRC cells. In this study, we first analyzed the expression of WTAP in CRC tissues and normal tissues using the GEPIA website, which includes 275 tumor tissues and 41 normal tissues. We found that WTAP expression was elevated in CRC tissues compared to normal tissues (**Figure 1A**). In addition, the patients with low WTAP expression had better overall survival (OS) by using GEPIA web server (**Figure 1B**). We further conducted a western blot analysis to confirm WTAP expression in 7 tumor samples and their adjacent normal tissues. The results indicated increased WTAP levels in CRC, consistent with the findings from GAPIA (**Figure 1C**, **1D**). Meanwhile, we examined WTAP expression in several CRC cell lines, as shown in **Figure 1E**. HCT-116, SW620 and SW480 showed higher levels of WTAP compared to HT-29, which is consistent with the analysis of WTAP mRNA levels (**Figure 1F**). Given the higher expression levels in HCT-116 and SW620, we selected these two cell lines to investigate the role of WTAP in CRC for this study.

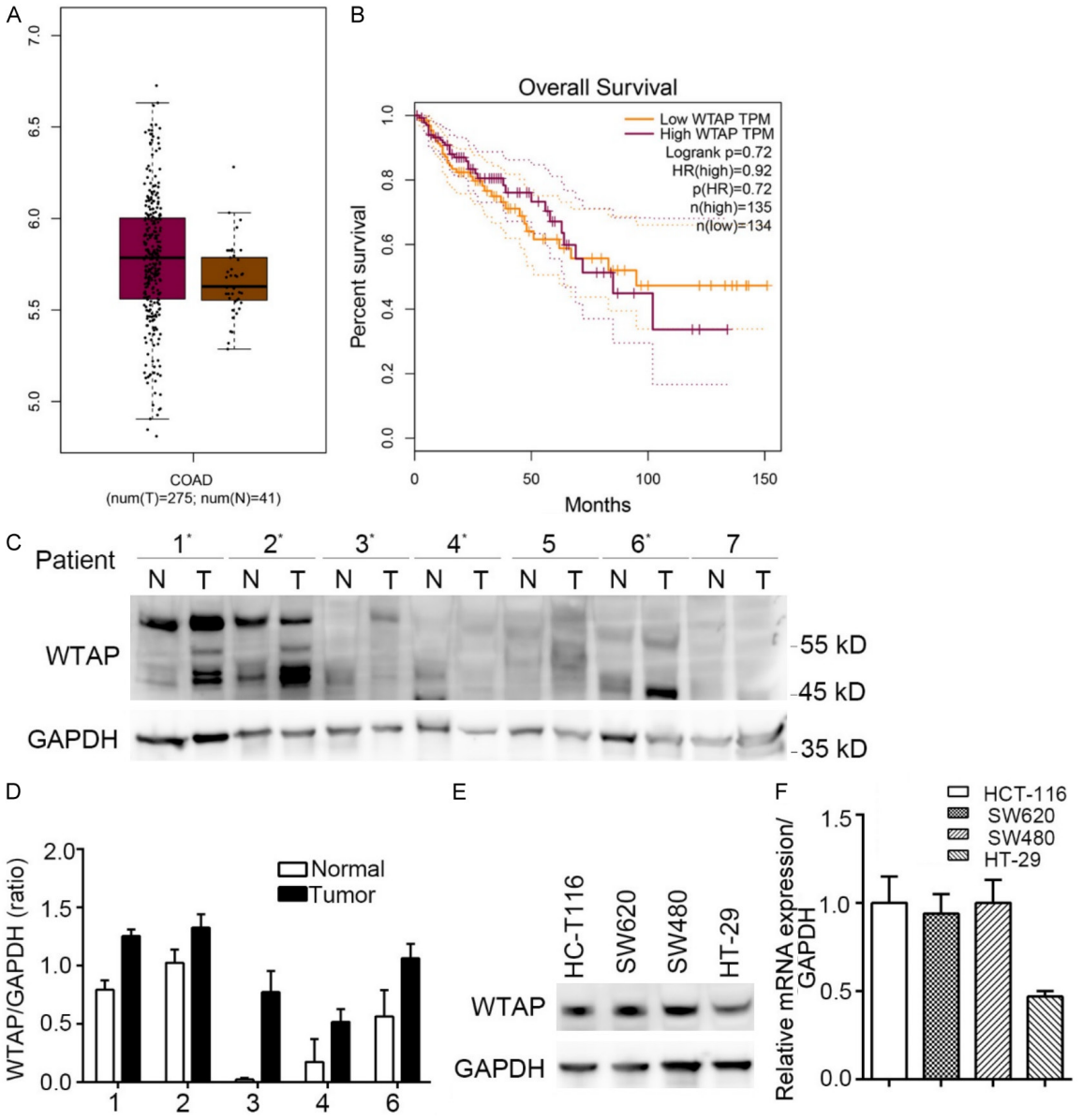

Figure 1. WTAP levels are elevated in CRC. A, B. The expression and prognostic role of WTAP were analyzed using the GEPIA website. C, D. WTAP expression was examined by western blot analysis in 7 CRC tissues and their adjacent normal tissues. E, F. Western blot analysis was performed to assess the expression of WTAP in CRC cell lines. WTAP, Wilms tumor 1-associated protein; GEPIA, Gene Expression Profiling Interactive Analysis; CRC, colorectal cancer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; N, normal tissues; T, tumor tissues; COAD, Colon adenocarcinoma.

WTAP knockdown inhibited sphere formation in HCT-116 and SW620 cells

To explore the role of WTAP in cell proliferation, we used siRNAs to knock down WTAP in CRC cells. Despite a 40% reduction in WTAP mRNA expression (**Figure 2C**), protein levels were reduced by 90% in HCT-116 cells (**Figure 2A**), with siWTAP-1 showing greater efficiency than siWTAP-2. Colony formation assays revealed that WTAP knockdown inhibited colony formation in HCT-116 cells (**Figure 2B**, **2D**). Similar results were obtained in SW620 cells (**Figure 2E-H**), where WTAP knockdown efficiency was confirmed by western blot and qRT-PCR analysis. WTAP knockdown by siWTAP-1 also inhibited colony formation in SW620 cells, while siWTAP-2 showed less impact. These findings suggest that WTAP plays a tumor-promoting role in CRC.

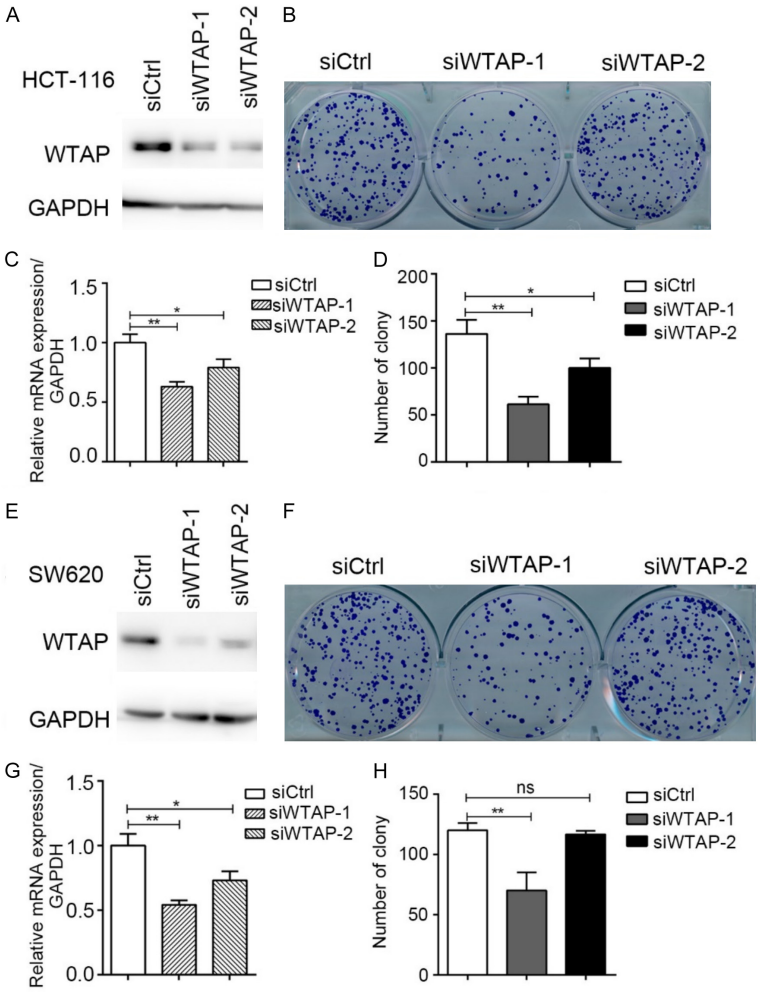

Figure 2. WTAP knockdown attenuated the proliferation of HCT-116 and SW620 cells. (A, E) The efficiency of WTAP knockdown was confirmed by western blot in HCT-116 (A) and SW620 (E) cells. (B, F) Colony formation assay of HCT116 (B) and SW620 (F) cells transfected with siCtrl or siWTAP. (C, G) mRNA levels of WTAP silenced by two siRNAs in HCT-116 (C) and SW620 (G) cells were determined by qRT-PCR. (D, H) Image J program was used to quantify the number of colonies. *, $P < 0.05$; **, $P < 0.01$; ns, not significant. WTAP, Wilms tumor 1-associated protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

WTAP knockdown suppressed cell migration by regulating SNAI1 expression

While previous studies have reported the function of WTAP in cell migration in cholangiocarcinoma and CRC [18, 19], the underlying mechanisms of WTAP in CRC cell migration are not fully understood. In this study, we found that WTAP knockdown repressed the migration of HCT-116 (**Figure 3A**) and SW620 (**Figure 3B**) cells. SNAI1 is a well-known regulator of tumor aggressiveness and metastasis through mediating the epithelial-to-mesenchymal transition (EMT) [20-22]. More importantly, we detected proteins related to cell migration and found that WTAP knockdown inhibited SNAI1 expression in HCT-116 and SW620 cells (**Figure 3C**, **3D**). The expression of SNAI1 was also decreased in both cell lines after WTAP knockdown by Immunofluorescent staining (IF) (**Figure 3E**, **3F**). Furthermore, WTAP knockdown increased CDH1 expression in SW620 but not in HCT-116 cells, and CDH2 expression did not change in either HCT-116 or SW620 cells upon WTAP knockdown.

SNAI1 expression was regulated by WTAP-mediated m6A modification

To investigate the role of WTAP in m6A modification in CRC cells, we first detected the total m6A level after WTAP knockdown in SW620 cells and found that m6A levels decreased in WTAP-knockdown SW620 cells compared to control cells (**Figure 4A**). We further predicted the m6A modification in *SNAI1* using the SRAMP prediction server (http://www.cuilab.cn/sramp) and identified one high-confidence site in *SNAI* (**Figure 4B**). Gene-specific m6A pull down assay and qRT-PCR analysis showed that the m6A levels of *SNAI1* increased in SW620 cells (**Figure 4C**). Additionally, there was a positive correlation between WTAP and SNAI1 expression based on data from GEPIA and cBioPortal (**Figure 4D**, **4E**).

Discussion

In this study, we investigated the role of WTAP in CRC cells. Although WTAP's roles have been previously explored, further evidence is needed to clarify its function in CRC. We examined the expression of WTAP in four CRC cell lines by western blot analysis and found that it was expressed in most cell lines. Importantly, we confirmed that WTAP expression was elevated

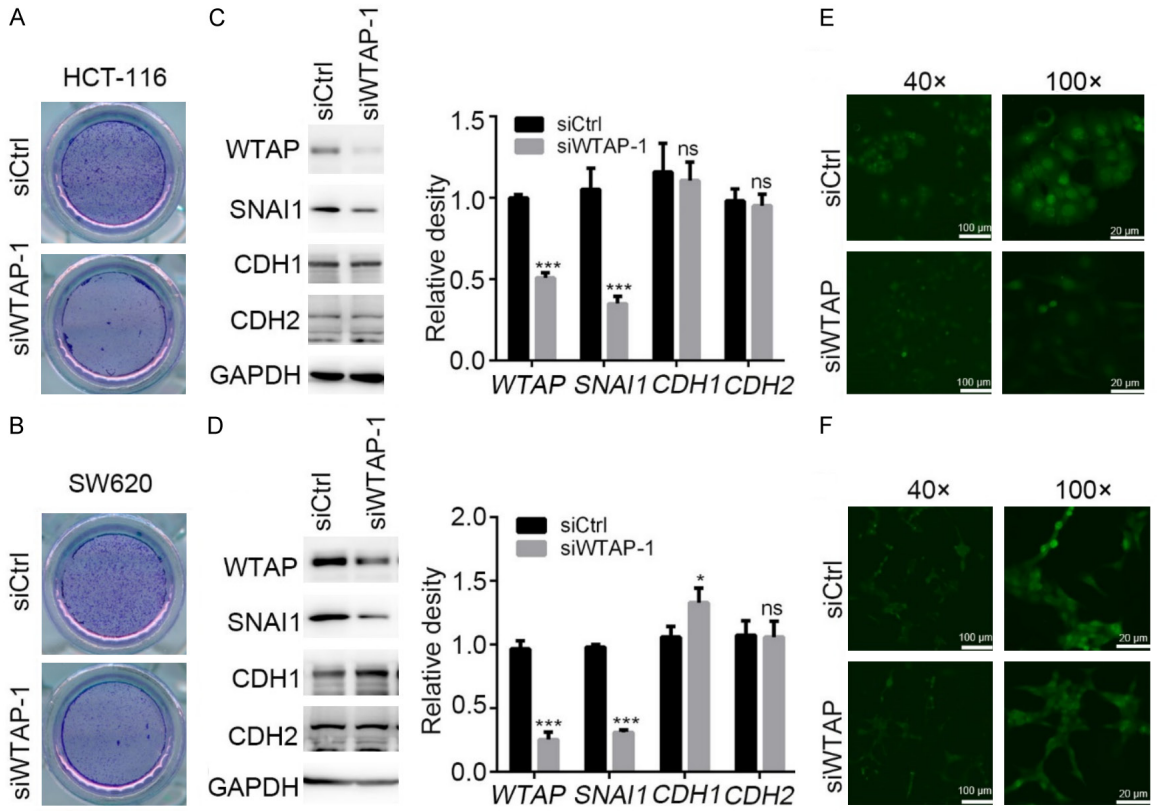

Figure 3. WTAP knockdown suppressed cell migration by decreasing SNAI1 expression in CRC cells. (A, B) Transwell migration assay of HCT-116 (A) and SW620 (B) cells transfected with siWTAP or control siRNA. (C, D) Western blot analysis of the expressions of CDH1, CDH2, and SNAI1 in HCT-116 (C) and SW620 (D) cells with or without WTAP silencing. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant. (E, F) HCT-116 (E) and SW620 (F) cells transfected with siWTAP were subjected to immunofluorescence (IF) staining with SNAI1 antibody. Scale bar: 100 μm. WTAP, Wilms tumor 1-associated protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CDH1, E-cadherin; CDH2, N-cadherin; SNAI1, Snail family transcriptional repressor 1.

in CRC tissues compared to their adjacent normal tissues. Moreover, low WTAP expression correlated with better overall survival. In vitro experiments demonstrated that WTAP downregulation inhibited colony formation, providing evidence that WTAP may function as an oncogene in CRC.

Furthermore, we observed a potential correlation between WTAP and SNAI1 in CRC. Western blot analysis revealed that WTAP deficiency impaired SNAI1 expression, thereby inhibiting cell migration. While the role of WTAP in cell migration has been reported in other studies, our analysis of the WTAP-SNAI1 correlation in CRC is novel. We further investigated this correlation using data from GEPIA and cBioPortal websites and confirmed the association between WTAP and SNAI1, though additional research is needed to elucidate the underlying mechanism.

Studies have revealed the role of WTAP in CRC tumorigenesis [18, 23, 24]. WTAP over-expression activates the *YTHDC1-VEGFA* axis to promote CRC development, especially angiogenesis [23]. WTAP regulates post-transcriptional repression of FLNA through m6A modification, with WTAP/FLNA inhibiting autophagy [24]. WTAP mediates the m6A modification of PDK4 to regulate CRC cell malignancy in vitro and in vivo [18]. In this study, we find WTAP increases SNAI1 expression through mediating its m6A modification, thereby promoting cell migration. These findings provide evidence that WTAP acts as a tumor promoter in CRC.

While our study demonstrates that WTAP mediates m6A modification of SNAI1, we did not further explore the specific "reader" (YTHDF1-3, YTHDC1 or YTHDC2) that identifies this m6A modification and mediates the effects of m6A-modified *SNAI1*. Overall, our study revealed th-

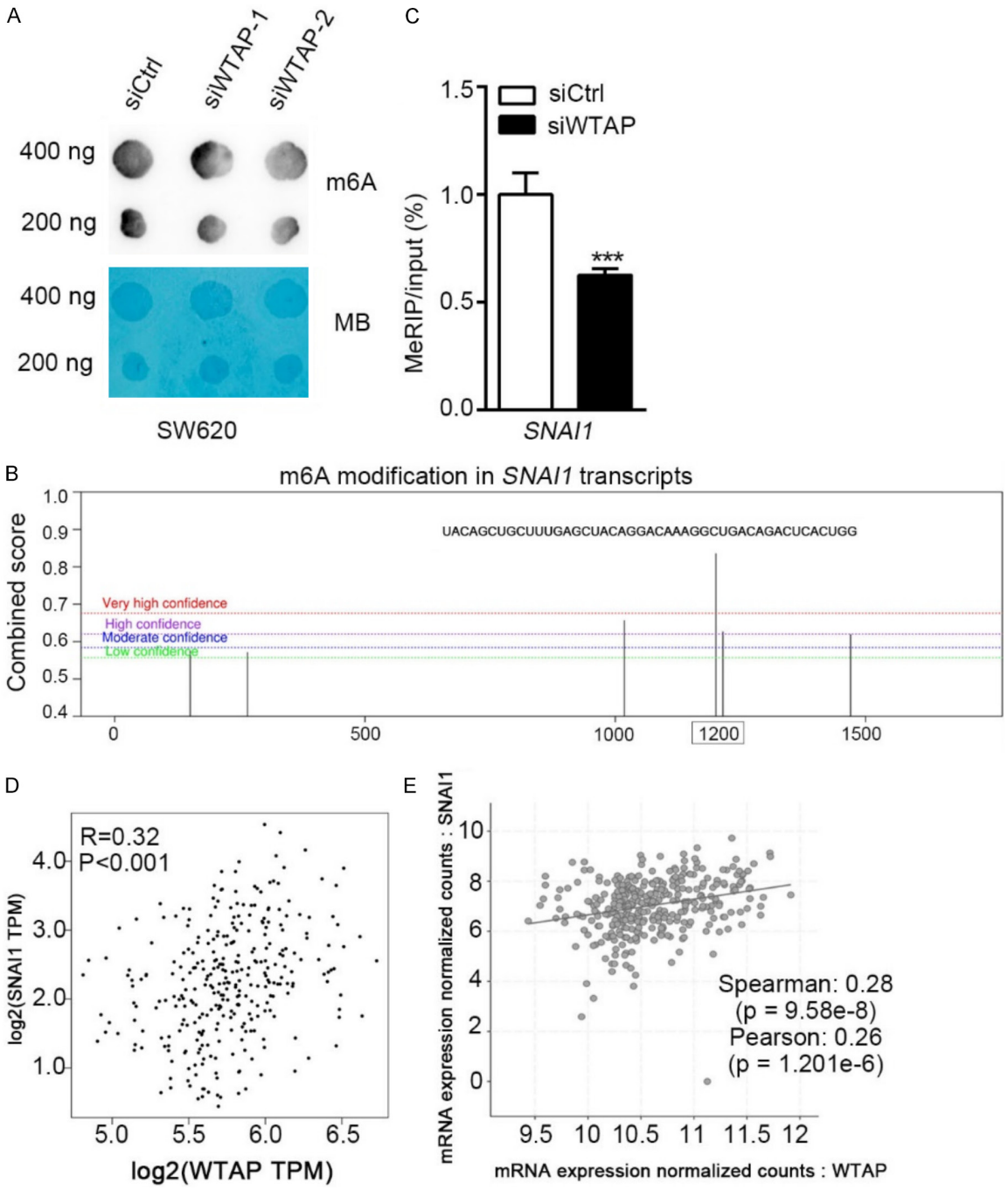

Figure 4. WTAP expression correlated with SNAI1 expression. A. RNA dot blot assessed m6A levels in WTAP-knockdown SW620 cells. B. Prediction of m6A modification sites in SNAI1 using the SRAMP prediction server. C. Gene-specific m6A qRT-PCR analysis of changes in m6A level in SNAI1 in SW620 cells. ***, P < 0.001. D. Analysis of the correlation between WTAP and SNAI1 expression using the GEPIA website. E. Exploration of the correlation between WTAP and SNAI1 in CRC using the Cbioportal dataset. WTAP, Wilms tumor 1-associated protein; m6A, N-6-methyladenosine; SNAI1, Snail family transcriptional repressor 1; GEPIA, Gene Expression Profiling Interactive Analysis.

at WTAP, acting as an oncogene, maintained SNAI1 expression through an m6A dependent mechanism in CRC cells, and indicated a potential biomarker panel for prognostic prediction in CRC.

Conclusions

WTAP is upregulated in CRC tissues, and its deficiency lead to decreased colony formation and migration of CRC cells. Additionally, high

WTAP expression is correlated with poor overall survival. Importantly, WTAP regulates cell migration by regulating SNAI1 expression in HCT-116 and SW620 cells, suggesting SNAI1 may be a downstream target of WTAP in CRC.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaowen Wen, Department of Colorectal Surgery, Shandong Cancer Hospital and Institute, Shandong First Medical University and Shandong Academy of Medical Sciences, No. 440, Jiyan Road, Huaiyin District, Jinan 250117, Shandong, China. E-mail: wenxiaiwen110@163.com

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