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TCF19 aggravates the malignant progression of colorectal cancer by negatively regulating WWC1

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Abstract. – OBJECTIVE: The aim of this study was to clarify the role of TCF19 in influencing the malignant progression of colorectal cancer (CRC) by negatively regulating WWC1.

PATIENTS AND METHODS: Relative expression levels of TCF19 and WWC1 in CRC tissues and cells were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The prognosis of CRC patients was assessed by the Kaplan-Meier method. Meanwhile, the correlation between TCF19 and pathological indexes of CRC patients was evaluated. Regulatory effects of TCF19/WWC1 on viability, colony formation ability, and migration in HT29 and HCT-8 cells were evaluated. Finally, rescue experiments were conducted to elucidate a negative feedback loop of TCF19/WWC1 in influencing the progression of CRC.

RESULTS: TCF19 was significantly up-regulated in CRC, while WWC1 was down-regulated. High-level TCF19 or low-level WWC1 indicated worse survival of CRC patients. Besides, TCF19 expression level was positively correlated with the occurrence of distant metastasis in CRC. Silence of TCF19 significantly attenuated proliferative and migratory capacities of HT29 cells. However, overexpression of TCF19 yielded the opposite trends in HCT-8 cells. WWC1 expression was negatively regulated by TCF19 in CRC tissues. In addition, knockdown of WWC1 abolished the regulatory effect of TCF19 on CRC cells.

CONCLUSIONS: TCF19 is closely correlated with the occurrence of distant metastasis and poor prognosis of CRC. Furthermore, it aggravates the malignant progression of CRC via negatively regulating WWC1.

Key Words:
TCF19, WWC1, Colorectal cancer (CRC), Malignant progression.

Introduction

Colorectal cancer (CRC) is a common malignant tumor characterized by high morbidity and mortality. Currently, CRC poses a great threat to human health and lives[1-3]. Mutations or loss of activities of tumor suppressor genes, as well as the activation of oncogenes are responsible for tumorigenesis of CRC[4,5]. Multiple biological markers have been identified to influence the prognosis and drug-sensitivity in CRC[6,7]. Meanwhile, these

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hallmarks are beneficial to develop individualized therapy for CRC patients and assess their prognosis[8,9].

The Human Genome Project has found approximately 23,000 coding genes in the human body, which are far fewer than transcribed proteins[10,11]. Over 90% of human genes exist RNA splicing, which is the reason for protein abundances[11]. Through precise gene regulation, the accurate response is reflected by the body under environmental stimuli. This may eventually help to maintain the homeostasis for energy supply[11,12]. During tumor progression, gene abnormalities are usually accompanied by changes in cancer-promoting transcripts[13,14]. With the popularity of second-generation sequencing technology, accumulating abnormal variable shears have been concerned[14]. As a novel tumor marker, abnormally expressed genes have been confirmed linked to pathways influencing tumor angiogenesis, metastasis, immune evasion, and cell metabolism[15].

In this study, bioinformatics was conducted to predict the possible differentially expressed genes in CRC. TCF19 was found differentially expressed in CRC tissues and adjacent normal tissues. TCF19 locates on human chromatin 6p21.2, which spans 5.69 kb and consists of 4 exons[16,17]. The aim of this investigation was to uncover the biological function of TCF19 in CRC, and to explore the underlying mechanism.

Patients and Methods

Patients and CRC Samples

CRC tissues and adjacent normal tissues were harvested from 41 CRC patients undergoing surgery. Tumor staging was assessed based on the guideline proposed by UICC. Baseline characteristics and follow-up data of CRC patients were recorded. Informed consents were obtained from patients and their families before the study. This research was approved by the Ethics Committee of The Affiliated Hospital of Inner Mongolia University for Nationalities.

Cell Culture

Normal colorectal mucosal cell line (FHC) and CRC cell lines (HCT-8, HCT-116, HT29) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS). HCT-116 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) and maintained in a 5% CO_2 incubator at 37°C. The cell passage was conducted at 80-90% of confluence.

Cell Transfection

Cells were first inoculated into 6-well plates and cultured for 70% of confluence. Cell transfection was conducted according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for subsequent functional experiments. Transfection plasmids were constructed by GenePharma (Shanghai, China).

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated into 96-well plates at a density of 2×10^3 cells/well. At day 1, 2, 3, and 4, respectively, the absorbance value at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

Colony Formation Assay

2.5×10^3 cells per well were inoculated into 6-well plates and cultured for 7 days. Subsequently, the cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.1% violet crystal for 30 min. After removing the staining solution and washing, formed colonies were aired dried. Finally, formed colonies were observed under a microscope, and the number of colonies was counted.

Transwell Assay

Cells were first seeded into 24-well plates at a density of 2.0×10^5/ml per well. 200 μL of cell suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate. Meanwhile, 500 μL of complete medium containing 10% FBS was added to the lower side. After 48 h of incubation, cells penetrated to the bottom side were fixed with methanol for 15 min and dyed with crystal violet for 20 min. Migrating cells were observed under a microscope. 5 fields of view were randomly selected for each sample, and the number of migrating cells was counted (magnification 20×).

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

Cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After purified by

DNase I treatment, extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using Primescript RT Reagent (TaKaRa, Otsu, Shiga Japan). Subsequently, obtained cDNA was subjected to qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as internal references. Each sample was performed in triplicate. Relative level of the gene was calculated by the $2^{-\Delta\Delta Ct}$ method. Primer 5.0 was used for designing qRT-PCR primers. Primer sequences used in this study were as follows: TCF19, F: 5'-CACTCAGACCCTCCGACTCT-3', R: 5'-CGATCGTGTAGGTCCGAGAAG-3'; WWC1, F: 5'-GGACGTAAACATGGGTCCTCTG-3', R: 5'-AGTGGTGTTGCGAGTCTCG-3'; U6: F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Total protein in cells was extracted and the protein concentration was determined. Protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in 5% skimmed milk for 1 h, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody for 2 h at room temperature. Then, the membranes were washed with 1×Tris-Buffered Saline and Tween-20 (TBST) for 1 min. Immunoreactive bands were finally exposed by the enhanced chemiluminescent substrate kit.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for data analyses. Experimental data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. The Kaplan-Meier curves were introduced for survival analysis, followed by log-rank test for comparing the differences between two curves. Spearman correlation test was performed to assess the relationship between expression levels of TCF19 and WWC1 in CRC tissues. $p<0.05$ was considered statistically significant.

Results

Upregulated TCF19 was Correlated with Distant Metastasis and Poor Prognosis in CRC

In comparison with adjacent normal tissues, TCF19 was significantly up-regulated in CRC tissues (Figure 1A). TCF19 was highly expressed in CRC cells when compared with normal colorectal mucosal cells (Figure 1B). Based on the median level of TCF19, enrolled 41 CRC patients were assigned to high or low-level TCF19 groups. The Kaplan-Meier curve indicated that significantly worse survival was observed in CRC patients of high-level group (Figure 1C). Subsequently, the correlation between baseline characteristics of CRC patients and TCF19 level was analyzed. As illustrated in Table I, TCF19 level was positively correlated with distant metastasis, rather than age, gender, tumor staging, and lymphatic metastasis of CRC patients.

TCF19 Accelerated the Proliferation and Migration of CRC

To elucidate the role of TCF19 in CRC, TCF19 overexpression or knockdown model was established in HCT-8 or HT29 cells, respectively. Transfection efficacies of si-TCF19 and pcDNA-TCF19 were tested by qRT-PCR (Figure 1D). In HT29 cells transfected with si-TCF19, significantly decreased viability, colony formation ability, and migration were observed (Figure 2A-2C). Conversely, HCT-8 cells overexpressing TCF19 exhibited remarkably promoted cell viability, colony formation ability, and cell migration (Figure 2A-2C). These results suggested that TCF19 accelerated the proliferation and migration of CRC.

TCF19 Negatively Regulated WWC1 Level

Both the protein and mRNA levels of WWC1 were significantly up-regulated in HT29 cells transfected with si-TCF19. Conversely, the protein and mRNA levels of WWC1 were down-regulated in HCT-8 cells overexpressing TCF19 (Figure 3A, 3C). TCF19 was downregulated in HT29 cells overexpressing WWC1, while was upregulated in HCT-8 cells transfected with si-WWC1 (Figure 3B, 3D). A negative correlation was identified between the expression levels of WWC1 and TCF19 in CRC tissues (Figure 3E). WWC1 was lowly expressed in CRC tissues and cell lines

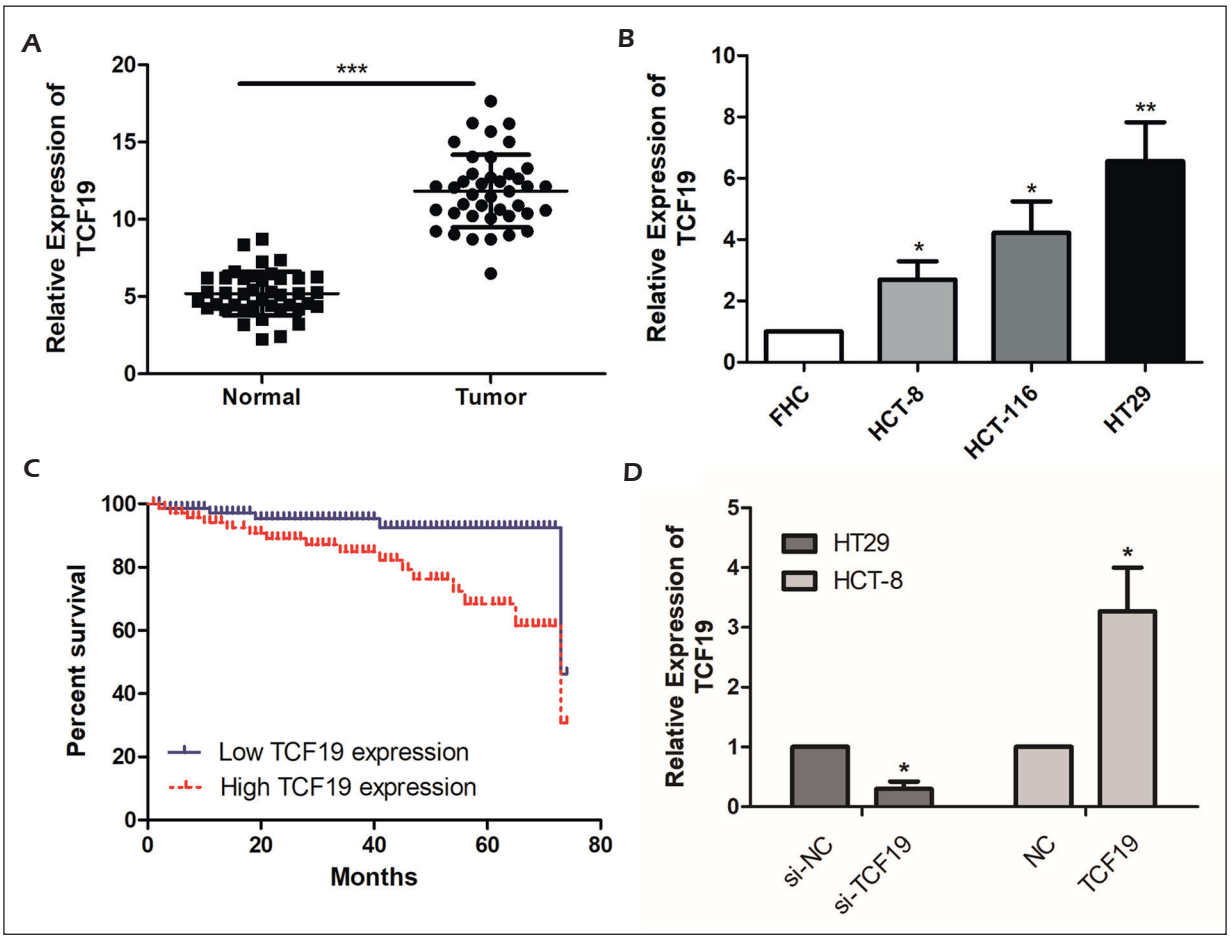

Figure 1. Upregulated TCF19 in CRC was linked to distant metastasis and poor prognosis. **A,** TCF19 level in CRC tissues and adjacent normal tissues. **B,** TCF19 level in normal colorectal mucosal cells (FHC) and CRC cells (HCT-8, HCT-116, HT29). **C,** Kaplan-Meier curves revealed survival in CRC patients with high and low expression level of TCF19. **D,** Transfection efficacy of si-TCF19 in HT29 cells and pcDNA-TCF19 in HCT-8 cells.

Table I. Association of TCF19 expression with clinicopathologic characteristics of colorectal cancer.

Parameters	No. of cases	TCF19 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.923
< 60	16	10	6	
≥ 60	25	16	9	
Gender				0.837
Male	20	13	7	
Female	21	13	8	
T stage				0.885
T1-T2	24	15	9	
T3-T4	17	11	6	
Lymph node metastasis				0.091
No	26	19	7	
Yes	15	7	8	
Distance metastasis				0.024
No	28	21	7	
Yes	13	5	8	

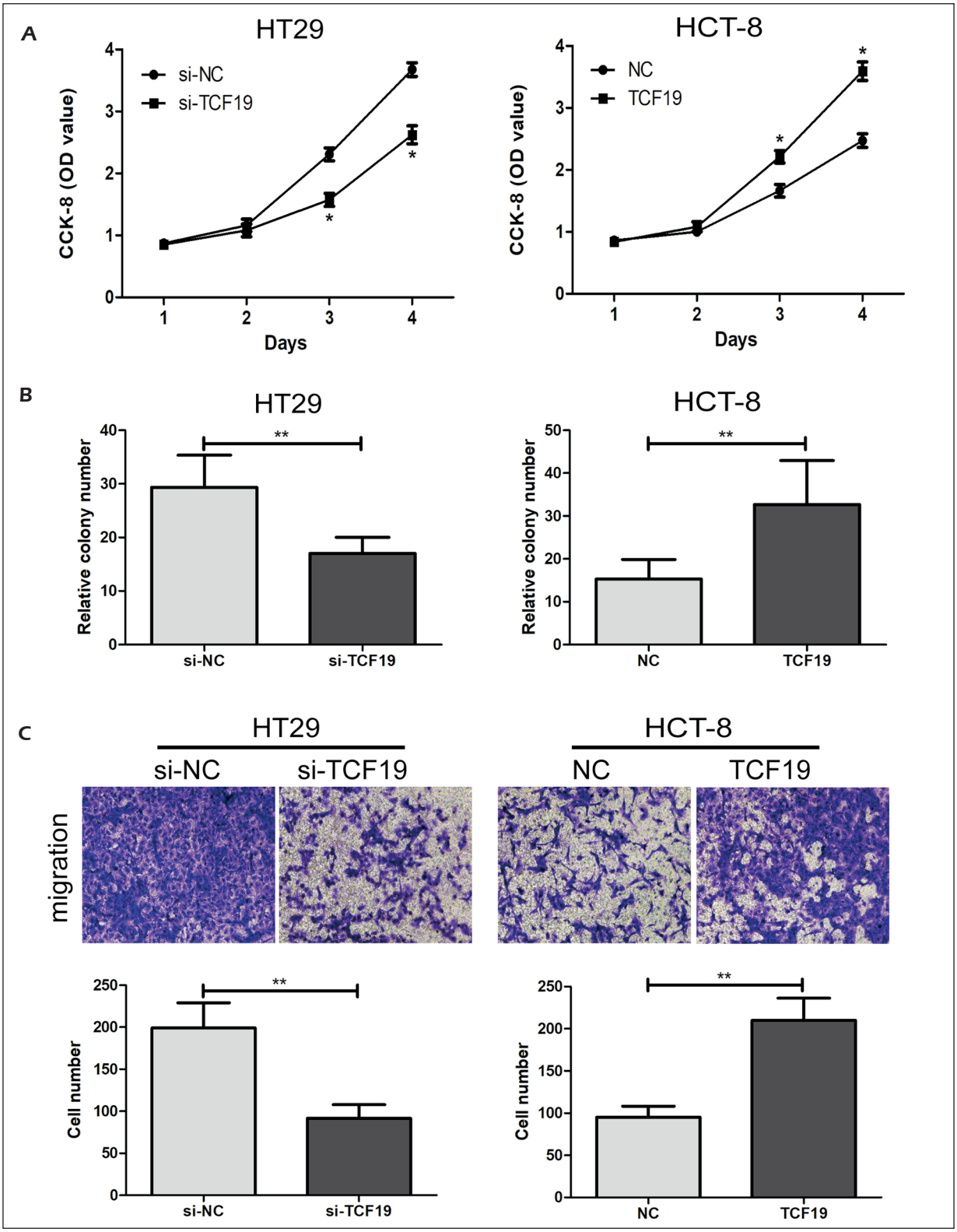

Figure 2. TCF19 accelerated the proliferation and migration of CRC. HT29 cells were transfected with si-NC or si-TCF19, and HCT-8 cells were transfected with NC or pcDNA-TCF19, respectively. **A,** Viability at day 1, 2, 3, and 4; **B,** Colony formation ability; **C,** Migratory ability (magnification 20×).

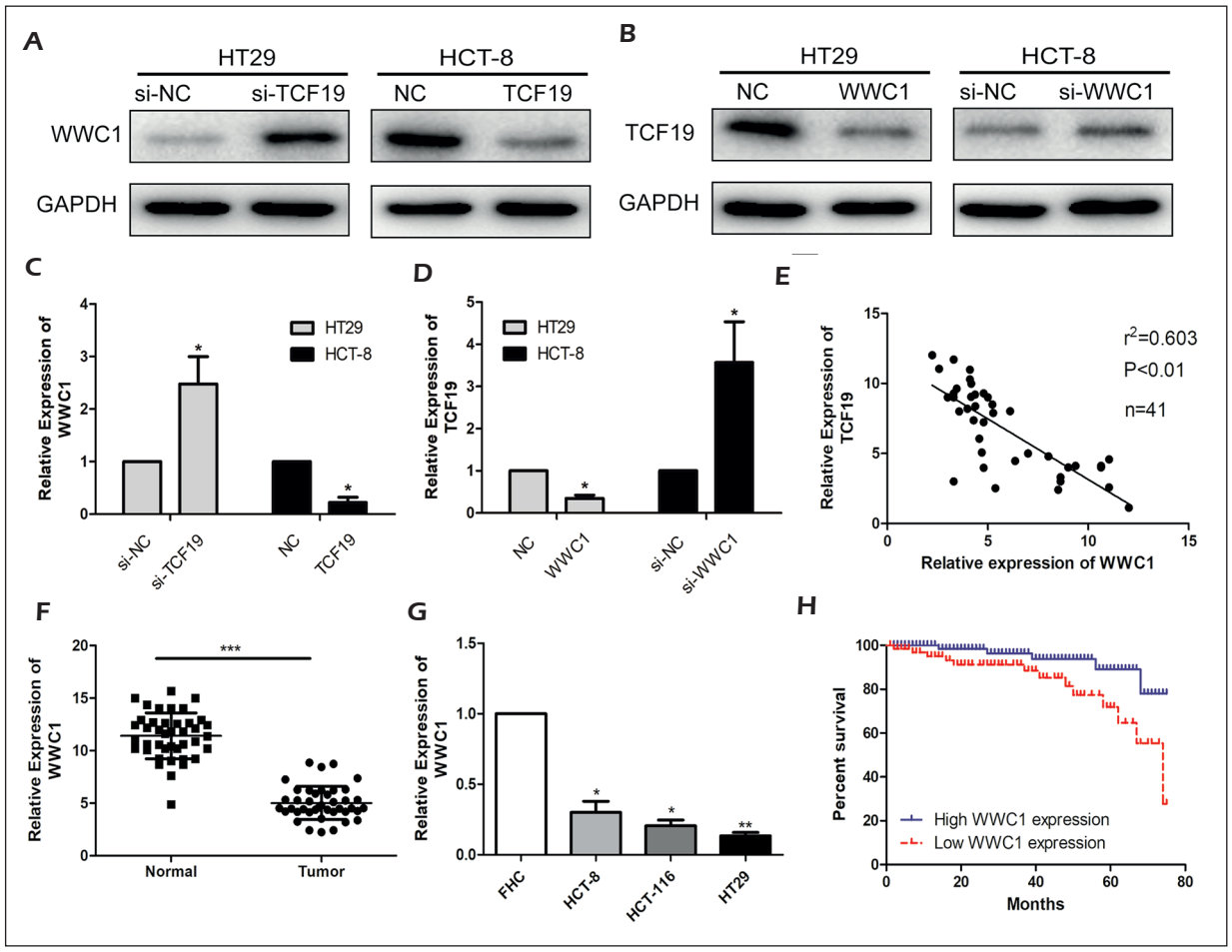

Figure 3. TCF19 negatively regulated WWC1 level. **A,** Protein level of WWC1 in HT29 cells transfected with si-NC or si-TCF19, and in HCT-8 cells transfected with NC or pcDNA-TCF19. **B,** Protein level of TCF19 in HT29 cells transfected with NC or pcDNA-WWC1, and in HCT-8 cells transfected with si-NC or si-WWC1. **C,** MRNA level of WWC1 in HT29 cells transfected with si-NC or si-TCF19, and in HCT-8 cells transfected with NC or pcDNA-TCF19. **D,** MRNA level of TCF19 in HT29 cells transfected with NC or pcDNA-WWC1, and in HCT-8 cells transfected with si-NC or si-WWC1. **E,** Negative correlation between expression levels of TCF19 and WWC1 in CRC tissues. **F,** WWC1 level in CRC tissues and adjacent normal tissues. **G,** WWC1 level in normal colorectal mucosal cells (FHC) and CRC cells (HCT-8, HCT-116, HT29). **H,** Kaplan-Meier curves revealed survival in CRC patients with high and low level of WWC1.

(Figure 3F, 3G). Furthermore, the Kaplan-Meier curves revealed that the survival of CRC patients with low level of WWC1 was significantly worse (Figure 3H).

WWC1 was Responsible for TCF19-Mediated Progression of CRC

To clarify the involvement of WWC1 in CRC progression influenced by TCF19, CRC cells were co-transfected with si-TCF19 and si-WWC1. Subsequently, changes in cellular behavior were observed. First, TCF19 level was up-regulated in CRC cells with WWC1 knockdown, which was reduced after co-silence of WWC1 and TCF19 (Figure 4A). Silence of WWC1 significantly accelerated the viability and colony formation ability of CRC cells. However, they could be reversed by knockdown of TCF19 (Figure 4B, 4C). Similarly, attenuated migratory ability in CRC cells with TCF19 knockdown was partially abolished by co-transfection of si-WWC1 (Figure 4D). A negative feedback loop TCF19/WWC1 was discovered to aggravate the progression of CRC.

Discussion

CRC is one of the most common malignancies in clinics. Due to the extensive application of colonoscopy and resection of polypoid adenomas,

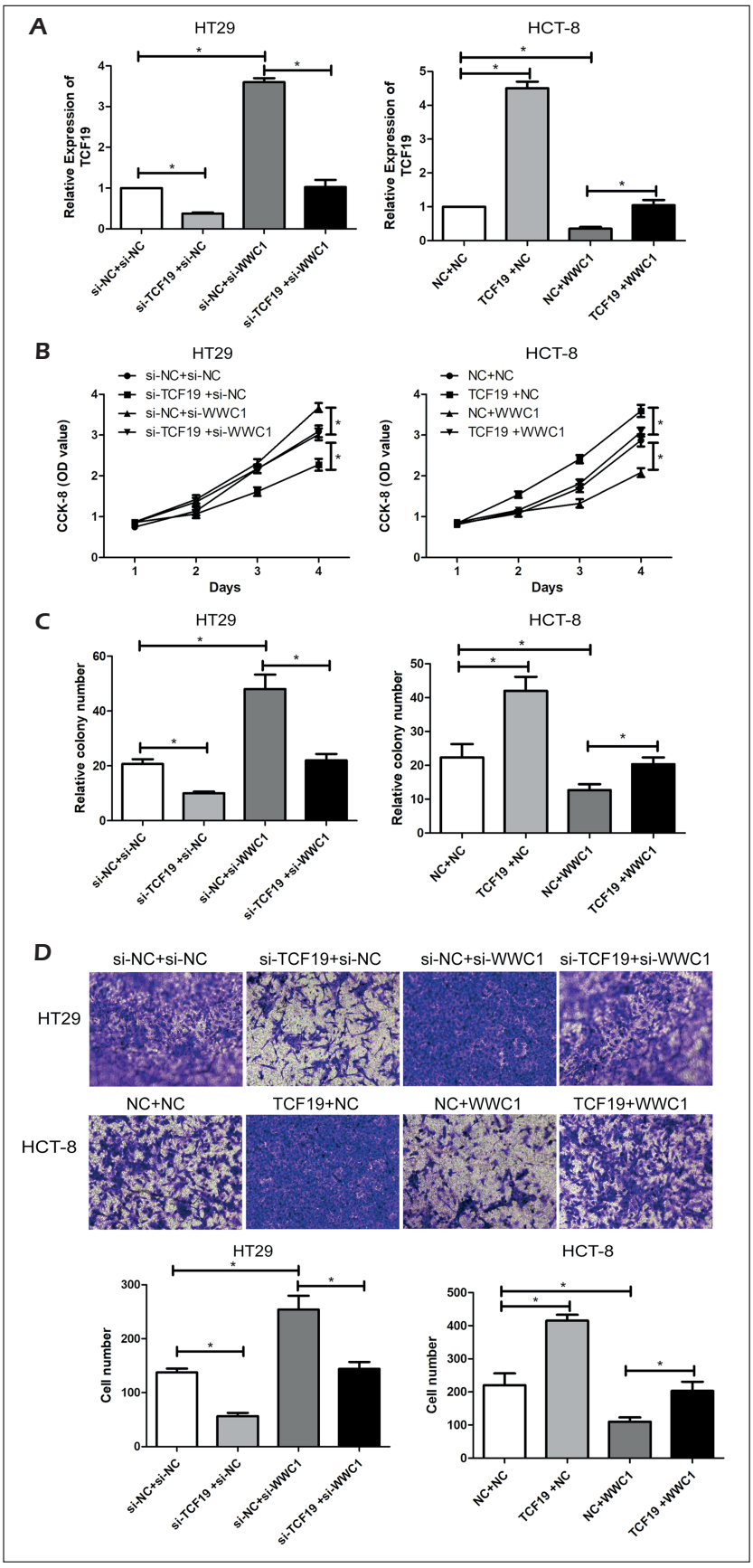

Figure 4. WWC1 was responsible for TCF19-mediated progression of CRC. HT29 and HCT-8 cells were transfected with si-NC, si-TCF19+si-NC, si-NC+si-WWC1, si-TCF19+si-WWC1, respectively. **A,** TCF19 level; **B,** Viability at day 1, 2, 3, and 4; **C,** Colony formation ability; **D,** Migratory ability (magnification 20×).

it is reported that the prevalence of CRC has decreased by 3% per year from 2004 to 2013. Nevertheless, the occurrence of CRC in males and females both ranks third in the United States[1-4]. Current studies[5,6] have found that the progression of CRC is complicated, involving multiple genes and pathways. Changes in precancerous lesions and related genes caused by cell biology dysfunction and growth pattern alteration are responsible for the occurrence of CRC[7]. Plenty of achievements have been made on investigating the mechanisms underlying the progression and metastasis of CRC[16,17].

Differentially expressed genes are widely involved in cell biology, including cellular behaviors, tissue specificity, organ formation, energy metabolism, and cellular immunity[14]. Therefore, these differentially expressed genes in pathological conditions may be utilized to monitor disease severity or develop target drugs. In recent years, they have been well concerned in tumor biology[11-13].

Several effective methods on altering gene expressions have already been developed, including inhibitor application, transgenics, and point mutations[15]. Transfection of vectors and application of inhibitors are mostly accepted approaches in changing target gene expressions, which are frequently applied in experiments[18-20]. TCF19 has been proved significantly up-regulated in many types of tumors. TCF19 level is closely associated with pathological features and clinical prognosis of tumor patients, suggesting an oncogenic role[18-20]. In this paper, TCF19 level in 41 pairs of CRC tissues and matched adjacent normal tissues was detected. The results indicated that TCF19 expression was markedly upregulated in CRC tissues. Meanwhile, a significant correlation between TCF19 level and distant metastasis of CRC patients was identified. In addition, *in vitro* experiments demonstrated the promotive effect of TCF19 on the viability and metastasis of CRC cells.

Bioinformatics and Dual-Luciferase reporter gene assay were conducted to search for the target gene of TCF19. Finally, WWC1 was screened out and selected. Overexpression of TCF19 markedly downregulated WWC1 level in CRC cells, verifying a negative correlation between expression levels of TCF19 and WWC1. Furthermore, WWC1 was found significantly downregulated in CRC tissues and cell lines. Survival analysis uncovered significantly worse prognosis of CRC patients with low level of WWC1 or high level of TCF19. Notably, knockdown of WWC1 abolished the role of TCF19 in influencing the proliferative and migratory abilities of CRC cells. Hence, a negative feedback loop was discovered that TCF19 aggravated CRC progression by negatively regulating WWC1.

Conclusions

We found that TCF19 level was closely correlated with the occurrence of distant metastasis and poor prognosis of CRC. Furthermore, it aggravated the malignant progression of CRC *via* negatively regulating WWC1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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