ORIGINAL ARTICLE

UDC: 575:616.348/.351-006-092
DOI: https://doi.org/10.2298/VSP250716076Z

# Inhibition of colorectal cancer cell proliferation, invasion, and migration, and induction of epithelial-mesenchymal transition *via TFF1* silencing

## Inhibicija proliferacije, invazije i migracije ćelija kolorektalnog karcinoma i indukcija epitelno-mezenhimalne tranzicije utišavanjem *TFF1* gena

**[1]Jing Zhao*, [1]Fan Wang*, Miao Li[†], Rui Liang[‡], Changying Li[§], Menglong Li*, Dilimulati Yalikun[‖], Zhiqiang Wang***

**The Second Hospital of Tianjin Medical University, *Department of Anorectal Surgery, [‡]Department of Pathology, [§]Tianjin Institute of Urology, Tianjin, China; [†]Baoding First Central Hospital, Department of Gynecology, Baoding, Hebei, China; [‖]The First Affiliated Hospital of Zhengzhou University, Department of Obstetrics and Gynecology, Zhengzhou, Henan, China**

**[1]The two authors contributed equally to this study.**

**Abstract**

**Background/Aim.** The identification of aberrant genes associated with colorectal cancer (CRC) and understanding their pathogenic mechanisms are important for early diagnosis and the determination of appropriate therapy. The aim of this study was to examine the impact of trefoil factor 1 (TFF1) on the proliferation, invasion, and migration of CRC cells. **Methods.** The expression of TFF1 in 40 CRC tissue samples was assessed using immunohistochemistry. Additionally, the expression of TFF1 in CRC cell lines HCT116 and Caco-2 was determined using real-time quantitative polymerase chain reaction (qPCR) and Western blot analysis. Then the Caco-2 cell line was transfected with a lentivirus-packaged small interfering ribonucleic acid to silence TFF1 expression. The cell counting kit-8 assay and cell colony formation assay were used to assess cell proliferation and colony formation capacity, while the Transwell assay and wound healing assay were used to assess cell invasion and migratory ability. Subsequently, the expression levels of E-cadherin and vimentin were assessed using qPCR and Western blotting to study the relationship between TFF1 expression and epithelial-mesenchymal transition. **Results.** The positive rate of TFF1 expression in CRC tissues was 35% (14/40). The HCT116 cell line demonstrated modest levels of TFF1 expression, while the Caco-2 cell line exhibited significant levels. Caco-2 cells expressed less TFF1 messenger ribonucleic acid and protein after *TFF1* silencing. The proliferative and colony-forming powers of Caco-2 cells, as well as their invasion and migratory capabilities, were subsequently seen to decrease. Furthermore, silencing *TFF1* expression led to an increase in E-cadherin expression and a decrease in vimentin expression. **Conclusion.** Caco-2 CRC cells can be inhibited from proliferating, migrating, and invading by silencing *TFF1* expression. TFF1 may play a significant role in the initiation, development, and metastasis of CRC by promoting epithelial-mesenchymal transition.

**Key words:**
**cell movement; cell proliferation; colorectal neoplasms; gene expression regulation; immunohistochemistry; trefoil, factor-1.**

**Apstrakt**

**Uvod/Cilj.** Identifikacija aberantnih gena povezanih sa kolorektalnim karcinomom (*colorectal cancer* – CRC) i razumevanje njihovih patogenih mehanizama je od značaja za ranu dijagnozu bolesti i određivanje odgovarajuće terapije. Cilj rada bio je da se ispita uticaj trefoil faktora 1 (TFF1) na proliferaciju, invaziju, i migraciju ćelija CRC. **Metode.** Ekspresija TFF1 u 40 uzoraka tkiva CRC procenjena je pomoću imunohistohemije. Pored toga, ekspresija TFF1 u CRC ćelijskim linijama HCT116 i Caco-2 određena je pomoću analize kvantitativne lančane reakcije polimerazom u realnom vremenu (*quantitative polymerase chain reaction* – qPCR) i *Western blot* analize. Zatim je Caco-2

**Correspondence to:** Zhiqiang Wang, The Second Hospital of Tianjin Medical University, Department of Anorectal Surgery, No. 23 Pingjiang Road, Hexi District, Tianjin 300211, China. E-mail: wzqcy20000@126.com; Dilimulati Yalikun, Department of Obstetrics and Gynecology, The First Affiliated Hospital of Zhengzhou University, No.1 Jianshe Dong Road, ErQi District, Zhengzhou 450002, Henan China. E-mail: 2410462725@qq.com

ćelijska linija transfektovana malom interferirajućom ribonukleinskom kiselinom upakovanom u lentivirus u cilju utišavanja ekspresije *TFF1*. Za procenu proliferacije ćelija i sposobnosti formiranja kolonija korišćeni su esej *cell counting kit-8* i test formiranja kolonija ćelija, dok su *Transwell* esej i test zaceljivanja rana korišćeni za procenu invazivne i migratorne sposobnosti ćelija. Nakon toga, nivoi ekspresije E-kaderina i vimentina procenjeni su pomoću qPCR i *Western blot* analize kako bi se ispitala veza između ekspresije TFF1 i epitelno-mezenhimalne tranzicije. **Rezultati.** Pozitivna stopa ekspresije TFF1 u tkivima CRC bila je 35% (14/40). U HCT116 ćelijskoj liniji detektovani su umereni nivoi ekspresije TFF1, dok su u Caco-2 ćelijskoj liniji zapaženi značajno viši nivoi ekspresije. U Caco-2 ćelijama zabeležen je smanjen nivo TFF1 informacione ribonukleinske kiseline i proteina nakon utišavanja *TFF1*. Zatim je uočeno da se smanjuje sposobnost proliferacije i formiranja kolonija kod Caco-2 ćelija, kao i njihove invazivne i migratorne sposobnosti. Štaviše, utišavanje ekspresije *TFF1* dovelo je do povećanja ekspresije E-kaderina i smanjenja ekspresije vimentina. **Zaključak.** Utišavanje ekspresije *TFF1* može inhibirati proliferaciju, migraciju i invaziju CRC ćelija Caco-2. Podsticanjem epitelno-mezenhimalne tranzicije TFF1 može igrati značajnu ulogu u nastanku, razvoju i metastaziranju CRC.

**Ključne reči:**
**ćelija, pokreti; ćelija, proliferacija; kolorektalne neoplazme; geni, regulacija ekspresije; imunihistohemija; trefoil, faktor-1.**

## Introduction

Colorectal cancer (CRC) is one of the most prevalent gastrointestinal malignancies, ranking third in terms of global incidence among malignant tumors and second in terms of mortality [1–3]. Recent advancements in next-generation sequencing and related technologies have facilitated the identification of genes with aberrant expression patterns in colorectal tumor tissues. Trefoil factor 1 (TFF1), a small polypeptide characterized by the presence of a trefoil factor domain, is primarily synthesized and secreted by mucous secretory epithelial cells lining the digestive tract, where it plays a pivotal role in mucosal damage repair [4]. Expression of TFF1 is ubiquitous at sites of mucosal injury throughout the gastrointestinal tract. Following gastrointestinal mucosal injury, the *TFF1* gene experiences a rapid up-regulation, thereby contributing to the process of gastrointestinal mucosal repair and reconstruction [5]. Several studies have shown that TFF1 expression is up-regulated in CRC tissues, and TFF1 levels have a wide variability (47.1–89%) [6–10]. The up-regulated expression demonstrated that TFF1 may play an important role in the tumorigenesis of human CRC.

Overexpression of TFF1 has been observed in various tumors, including breast cancer, pancreatic cancer, pulmonary cancer, and bladder cancer. Its overexpression has been associated with tumor cell proliferation, apoptosis, metastasis, and angiogenesis [11–14]. However, the involvement of TFF1 in the pathogenesis and progression of CRC remains a subject of debate.

The aim of this study was to examine the expression levels of TFF1 in CRC and elucidate its biological functions and potential underlying mechanisms in this specific cancer type.

## Methods

### *Research material*

A total of 40 patient-derived CRC tissue specimens were collected, comprising 22 cases of colon cancer and 18 cases of rectal cancer in 2022. Notably, none of the patients had received neoadjuvant therapy, and there was no prior medical history of other malignancies. This study was approved by the Ethics Committee of the Second Hospital of Tianjin Medical University (No. KY2023K207, from October 26, 2023). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

RPMI-1640 medium and fetal bovine serum were acquired from Gibco (USA). Penicillin-streptomycin solution was obtained from Invitrogen (USA). Polyvinylidene fluoride membrane was purchased from Millipore (USA), and Matrigel was acquired from Corning (USA). Secondary antibodies were obtained from Cell Signaling Technology (USA). Sterile petri dishes, 6-well plates, and 96-well plates were purchased from Corning (USA). SYBR Green Fast quantitative real-time polymerase chain reaction (qPCR) Mix was purchased from Roche (Basel, Switzerland). The reverse transcription kit was purchased from Thermo (USA). The quantitative reverse transcription-PCR primers were designed by Shanghai Sangon Biological Engineering Co., Ltd. (China). The TFF1 antibody was supplied by Shanghai Santa Cruz Biotechnology Company (China). The marker for Western blot protein staining and Lipofectamine® 2000 were acquired from Invitrogen (USA). The packaging of small interfering ribonucleic acid (siRNA) and lentivirus was provided by Shanghai Jima Gene Co., Ltd. (China). The cell counting kit-8 (CCK-8) was acquired from Xi'an Mishu Biotechnology Co., Ltd. The human CRC cell line HCT116 and normal colon epithelial cell line CCD-18Co were provided by Zunyi Medical University, while the CRC cell line Caco-2 was obtained from Shanghai Zhongqiao Xinzhou Biological Co., Ltd.

### *Immunohistochemical analysis*

The expression of TFF1 was detected using the streptavidin-peroxidase method. CRC tissues and adjacent non-tumor tissues were embedded in paraffin and sectioned. The tissue sections were baked at 60 °C for 1 hr, subsequently rewarmed to room temperature, and dewaxed in xylene for 30 min. After alcohol rehydration treatment, the tissue sections were washed three times with phosphate-buffered saline (PBS), then subjected to high-temperature

antigen retrieval using ethylenediaminetetraacetic acid, and again washed three times with PBS. The samples were then placed in a humidified chamber, where endogenous peroxidase activity was blocked with 4% $H_2O_2$ for 10 min at room temperature, followed by three PBS washes. Non-specific binding was blocked with 5% bovine serum albumin at 37 °C for 1 hr. The TFF1 antibody was appropriately diluted according to the manufacturer's instructions. Following the application of the primary antibody, the slides were incubated overnight at 4 °C, rinsed three times with PBS, treated with the secondary antibody, and then incubated at room temperature for 1 hr. After three additional washes with PBS, a 3,3'-diaminobenzidine color solution was applied, and the samples were incubated at room temperature for 5 min. Finally, the slides were counterstained with hematoxylin, dehydrated, cleared, and sealed. The scoring was performed as follows: under the microscope, the degree of cell staining and the proportion of positive cells were scored, respectively. Degree of cell staining: 0 – no coloration, 1 – light yellow, 2 – yellow, and 3 – brown. Proportion of positive cell scoring: ≤ 10% – 1 point, 10% to 50% – 2 points, 50% to 80% – 3 points, and ≥ 80% – 4 points. The degree of cell staining and the rate of positive cells were multiplied, and the result ≤ 1 was considered negative (-), 2–4 indicated weak positive (+), 5–8 positive (++), 9–12 strong positive (+++). Histomorphological analysis was performed using an Olympus BX53 microscope (Olympus Corporation, Tokyo, Japan) at a magnification of ×200. CellSens software (version 2.3, Olympus) was used for image acquisition. Five sections *per* tissue were analyzed.

*Cell culture*

The CRC cell lines HCT116 and Caco-2, as well as the normal colonic epithelial cell line CCD-18Co, were maintained in petri dishes with RPMI-1640 medium, 10% fetal bovine serum, and 1% penicillin–streptomycin solution at 37 °C in a humidified incubator containing 5% $CO_2$.

*Quantitative real-time polymerase chain reaction*

Total RNA was extracted using the TRIzol reagent (Invitrogen, USA), and reverse-transcribed into complementary DNA (cDNA) using a qPCR kit with the following primers: glyceraldehyde-3-phosphate dehydrogenase primer: 5′-ACA ACTTTGGTATCGTGGAAGG-3′(forward), 5′-GCCATCAC GCCACAGTTTC-3′(reverse), and TFF1 primer: 5′-CCCTC CCAGTGTGCAAATAGG-3′(forward), 5′-GAACGGTGTCG TCGAAACAG-3′(reverse).

For reverse transcription, 0.5 μg of total RNA was mixed with 5 μL of oligo deoxythymidine primer in a 200 μL ribonuclease (RNase)-free tube and brought to a final volume of 11.5 μL with RNase-free water. The mixture was heated at 70 °C for 5 min and then chilled on ice for 5 min. After brief centrifugation, 5 μL of 5 × avian myeloblastosis virus (AMV) buffer, 2.5 μL of deoxyribonucleoside triphosphates, 0.5 μL of RNase inhibitor, and 0.5 μL of AMV reverse transcriptase were added, and the volume was brought to 25 μL with RNase-free water. The reaction was carried out at 42 °C for 60 min to synthesize cDNA. For qPCR, the reaction system contained 20 μL of Hieff qPCR SYBR Green Master Mix, 10 μL of RNase-free water, 3 μL of forward primer, 3 μL of reverse primer, and 4 μL of cDNA template. After brief centrifugation, reactions were amplified under standard cycling conditions. The following PCR cycling conditions were used: 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s.

*Western blotting analysis*

Total protein was extracted using a protein extraction reagent, and the concentration of total protein was determined using the Bradford method. The proteins were then transferred onto a polyvinylidene fluoride membrane. After incubating with 5% skim milk, the membranes were incubated with primary and secondary antibodies. Finally, the protein bands were exposed and developed using an enhanced chemiluminescence system. The gray value of target bands was analyzed using ImageJ software.

*Small ribonucleic acid transfection*

The cellular samples were divided into three groups: the negative control (NC) group (non-targeting siRNA), the small interference transfection Group I (siRNA1 group), and the small interference transfection Group II (siRNA2 group). Caco-2 cells ($1.0 \times 10^5$ cells/mL) were cultured in 6-well plates, and transfection was started when the cells were in good condition and had reached 80–90% cell density. Experiments were performed according to the Roche X-tremeGENE siRNA transfection kit. Under sterile conditions, 2 μg of the siRNA transfection sequence was mixed with 100 μL of Opti-MEM I. Separately, 10 μL of the transfection reagent was diluted in 100 μL of Opti-MEM I and incubated for 5 min at room temperature. The solutions were then mixed and incubated for 15 min at room temperature. Finally, the mixture was added to the 6-well plates. The volume was brought to 2 mL with culture medium and incubated at 37 °C with 5% $CO_2$.

The siRNA target sequence for TFF1 is as follows: siRNA1: 5′-AGUGGGUAUGUUUGGUAGGTT-3′, siRNA2: 5′-AUGGUAUAAGGAUAGAAGCTT-3′. After 72 hrs of transfection, RNA and protein were extracted and examined using Western blotting and qPCR to compare the silencing efficiency of the two short interfering RNA fragments.

*Lentiviral transfection*

The cellular specimens were divided into three groups: the blank group (human CRC cells without siRNA vehicle and medium), the NC short hairpin (sh)RNA group (CRC cells transfected with empty vehicle), and the TFF1 shRNA group (CRC cells transfected with specific TFF1 siRNA). Cells in good growth condition were selected and seeded in 6-well plates ($5 \times 10^5$ cells/well) and incubated overnight. When the cell growth density reached 40–50%, the cells

were ready for transfection. For viral infection, 400 μL of complete medium containing polybrene (final concentration, 5 μg/mL) was prepared, and 20–100 μL of lentiviral stock solution was added. The culture medium in 6-well plates was replaced with the viral suspension, and blank and NC groups were established in parallel. After 24 hrs of incubation, the viral medium was replaced with fresh complete medium, and cells were maintained under standard culture conditions. The lentivirus carried a green fluorescent protein reporter gene, and transduction efficiency was monitored by fluorescence microscopy. When the proportion of fluorescent-positive cells reached 80%, the cells were either passaged or cryopreserved. To establish stable cell lines, untransduced cells were eliminated by puromycin selection using the resistance gene encoded by the viral vector. Subsequent experiments were performed once cells had recovered and reached optimal growth status.

*Cell counting kit-8 assay*

Cell proliferation capacity was evaluated by CCK-8 assay. Cells from each group were suspended at a density of $1 \times 10^4$ cells/mL, and 100 μL/well of the suspension was seeded into 96-well plates in triplicate. Measurements were taken at 0, 24, 48, 72, and 96 hrs. Ten microliters of CCK-8 solution was added to each well and incubated for 2 hrs at 37 °C. Subsequently, the optical density value was measured with a microplate reader (Varioskan LUX, Thermo Fisher Scientific).

*Cell colony formation assay*

Exponentially growing cells were harvested, counted, and seeded into 6-well plates at a density of 500 cells/well. After gentle shaking to evenly distribute the cells, the plates were incubated at 37 °C with 5% $CO_2$ for 10–14 days, during which the medium was replaced every 3 days. After cell clones became visible, the culturing process was concluded. The cells were then fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. The number of cell colonies was subsequently quantified. Colonies containing ≥ 50 cells were counted under an optical microscope (Olympus BX53, Tokyo, Japan), and images were acquired with the DP74 imaging system using cellSens dimension software (version 2.3, Olympus).

*Transwell assay*

Matrigel was diluted with precooled RPMI-1640 culture medium at a 1 : 3 ratio. The mixture was then spread over the upper surface of the Transwell chamber. After incubating at 37 °C for 1 hr, 750 μL of RPMI-640 medium was added to the lower chamber. The transfected cells were suspended at a concentration of $1 \times 10^5$ cells/mL. Subsequently, 200 μL of the cell suspension was seeded into the upper layer of the Transwell chamber and cultured at 37 °C for 72 hrs. Cells that successfully traversed the membrane were stained with a 0.1% crystal violet solution, and the number of cells was counted using an inverted microscope.

*Wound healing assay*

Cell migratory ability was analyzed using a wound healing assay. The transfected cells were seeded in a 6-well plate. When the cell density reached 90%, traces were drawn using a 200 μL tip head. Photographs of the same wound site were taken at 0, 24, 48, and 72 hrs, and the migratory distance was measured using ImageJ software.

*Expression of E-cadherin and vimentin*

The expressions of E-cadherin and vimentin messenger (m)RNA and protein in Caco-2 cells were assessed using qPCR and Western blot. The techniques for qPCR and Western blot were identical to those previously described.

*Statistical analysis*

The statistical software SPSS version 25.0 was employed. The measurement results are reported as the mean ± standard deviation, and the *t*-test and analysis of variance were used to compare the groups. The value of $p < 0.05$ was considered statistically significant.

**Results**

*Trefoil factor 1 expression in colorectal cancer tissues*

Forty CRC tissue sections were collected and examined by immunohistochemistry staining. Results revealed that TFF1 expression was predominantly localized in the cytoplasm of cells in colorectal cancer tissues (Figure 1A), while no obvious TFF1 expression was detected in normal colonic epithelial tissues (Figure 1B) or in the negative control without primary antibody (Figure 1C). The positive rate of TFF1 expression in CRC tissues was determined to be 35% (14/40).

*Trefoil factor 1 expression in colorectal cancer cell lines*

Western blot and qPCR were used to compare the expression of the *TFF1* gene protein and mRNA in the CRC cell lines HCT116 and Caco-2, as well as the normal epithelial cell line CCD-18Co. The expression of TFF1 protein and mRNA in Caco-2 was higher than that in HCT116 cells and CCD-18Co ($p < 0.05$) (Figure 2A, B, and C). Therefore, Caco-2 cells were adopted for further investigation.

*Inhibiting trefoil factor 1 expression*

After being transfected with two siRNA sequences, the TFF1 expression in Caco-2 cells was detected by qPCR. The results revealed a significant decrease in the mRNA expression levels in the siRNA1 and siRNA2 groups compared to the control group ($p < 0.05$). Moreover, siRNA2 inhibited TFF1 expression more effectively than siRNA1. The siRNA2 sequence was therefore packaged into a lentivirus and

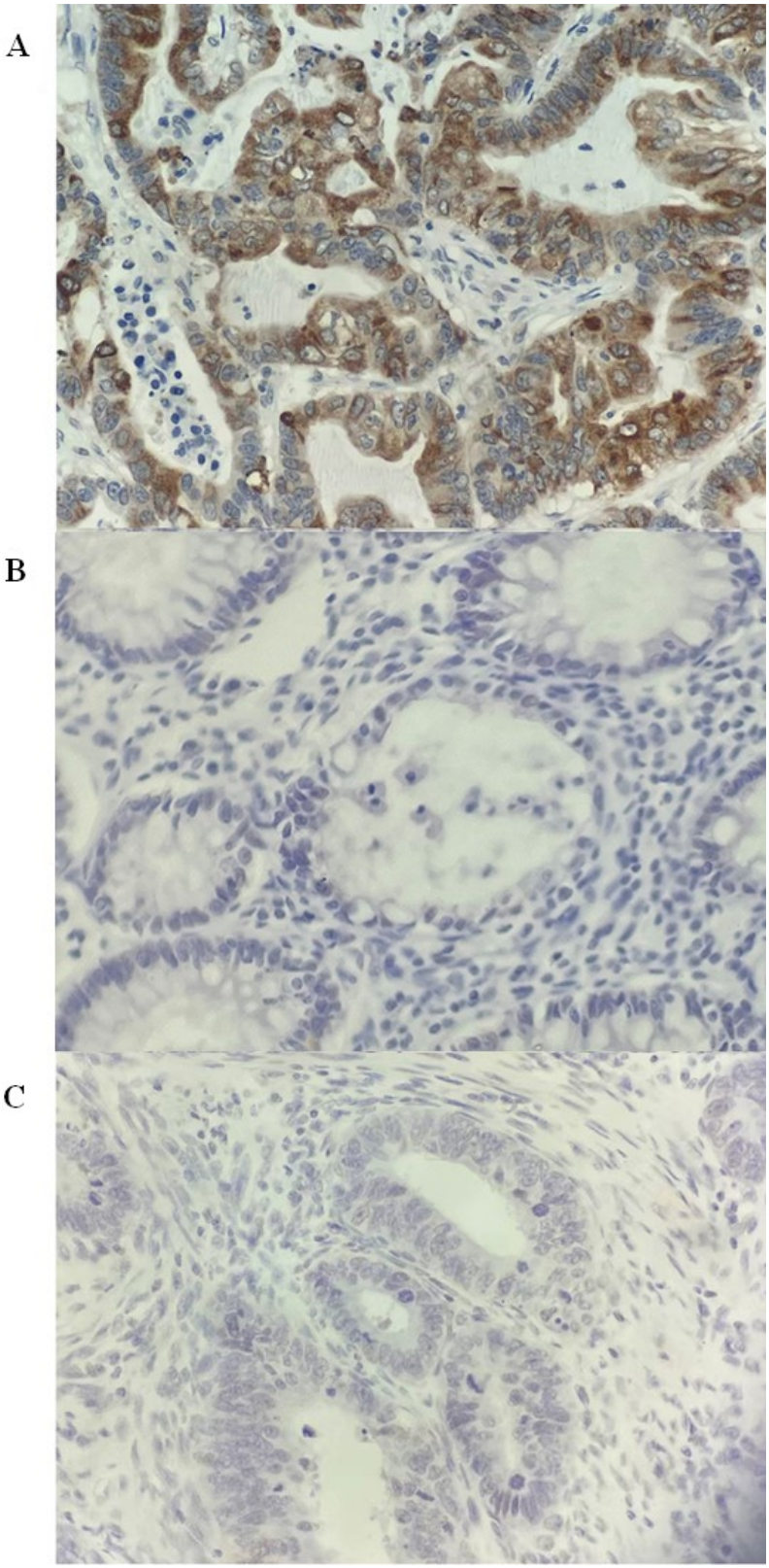


**Fig. 1 – Expression of trefoil factor 1 (TFF1) protein in colorectal cancer tissues: A) positive immunohistochemistry (IHC) expression of TFF1 in colon cancer tissues (×200); B) negative IHC expression of TFF1 in normal colon epithelial tissues (×200), and C) negative IHC expression of TFF1 in normal colon epithelial tissues without primary antibody (×200).**

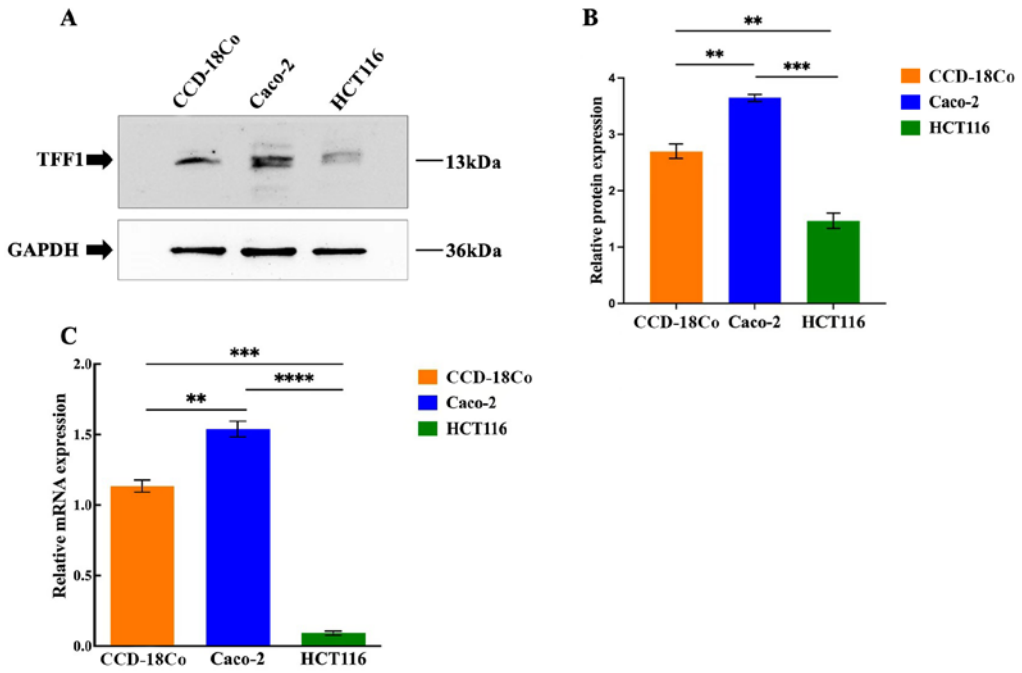


**Fig. 2 – Trefoil factor 1 (TFF1) expression in colorectal cancer cell lines: A) Western blotting assay of TFF1 protein expression; B) grayscale analysis of Western blot results, and C) TFF1 mRNA expression detected by qPCR. GAPDH – glyceraldehyde-3-phosphate dehydrogenase; kDa – kilodalton; mRNA – messenger ribonucleic acid; qPCR – quantitative polymerase chain reaction.**
**Results are presented as mean ± standard deviation derived from triplicate analyses. *$p < 0.05$.**

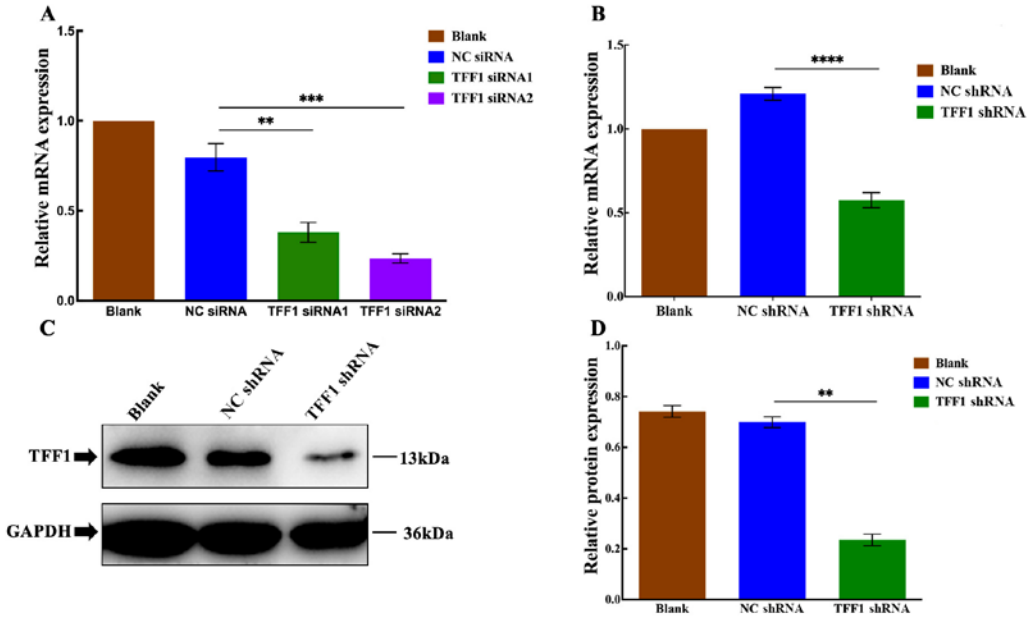


**Fig. 3 – TFF1 mRNA and protein expression in cells after transfected with interfering siRNA sequences: A) qPCR detection of TFF1 mRNA after siRNA transfection; B) qPCR detection of TFF1 mRNA after lentiviral shRNA infection; C) Western blotting analysis of TFF1 protein expression, and D) relative TFF1 protein expression analyzed by grayscale scanning of Western blot bands.**
**NC – negative control; siRNA – small interfering ribonucleic acid; shRNA – short hairpin ribonucleic acid. For other abbreviations, see Figure 2.**
**Results are expressed as mean ± standard deviation of three independent experiments. *$p < 0.05$.**

used to infect Caco-2 cells in subsequent experiments. The efficiency of lentivirus infection was validated using Western blot and qPCR. The TFF1 protein and mRNA levels demonstrated a substantial drop in the TFF1 shRNA group compared to the control group ($p < 0.05$) (Figure 3A, B, C, and D).

*Proliferation ability analysis*

Compared to the control group, the knockdown of TFF1 dramatically decreased CRC cell activity and diminished proliferative capacity ($p < 0.05$) (Figure 4A). Correspondingly, the results of the colony formation assay demonstrated a suppression of the colony formation ability in TFF1-knockdown cells ($p < 0.05$) (Figure 4B and C).

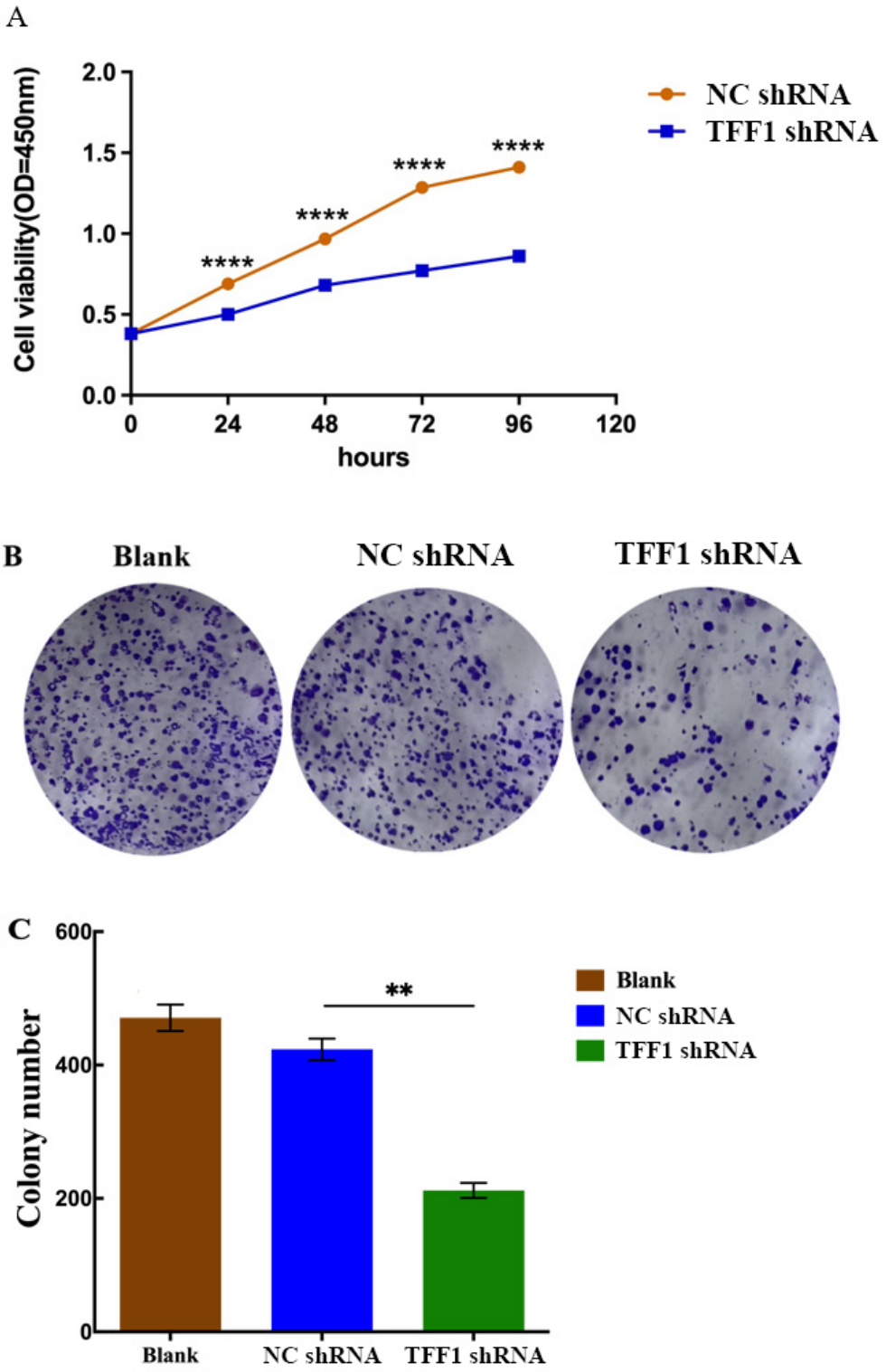


**Fig. 4 – Proliferation ability analysis: A) cell counting kit-8 assay depicting cell viability [optical density (OD) = 450 nm]; B) representative images of colony formation assay (crystal violet staining, magnification ×200), and C) quantitative analysis of colony numbers from the colony formation assay.**
**For abbreviations, see Figures 1 and 3.**
**Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.**

*Invasion ability analysis*

Cell invasion ability was evaluated using the Transwell assay. After culturing for 72 hrs, the number of transmembrane cells in the blank group, the NC shRNA group, and the TFF1 shRNA group was 167.329 ± 6.560, 143.190 ± 4.863, and 35.610 ± 5.728, respectively. The number of transmembrane cells in the TFF1 shRNA group was significantly lower than that in the blank and NC shRNA groups (all $p < 0.01$) (Figure 5A and B).

*Migratory ability analysis cell*

The migratory capacity of CRC cells was explored through a wound healing assay. The results demonstrated a substantial decrease in the migratory rate of the TFF1 shRNA group compared to the control group ($p < 0.05$) (Figure 6A and B). This result suggests that TFF1 knockdown reduces the migratory ability of CRC cells.

*Relationship between trefoil factor 1 expression and epithelial-mesenchymal transition*

The epithelial-mesenchymal transition (EMT) markers E-cadherin and vimentin were detected by qPCR and Western blotting analysis. The results demonstrated that the mRNA and protein expression levels of E-cadherin increased while the mRNA and protein expression levels of vimentin decreased following interference with *TFF1* gene expression (Figure 7A and B). These data imply that TFF1 is integrally involved in and enhances the EMT pathway in CRC.

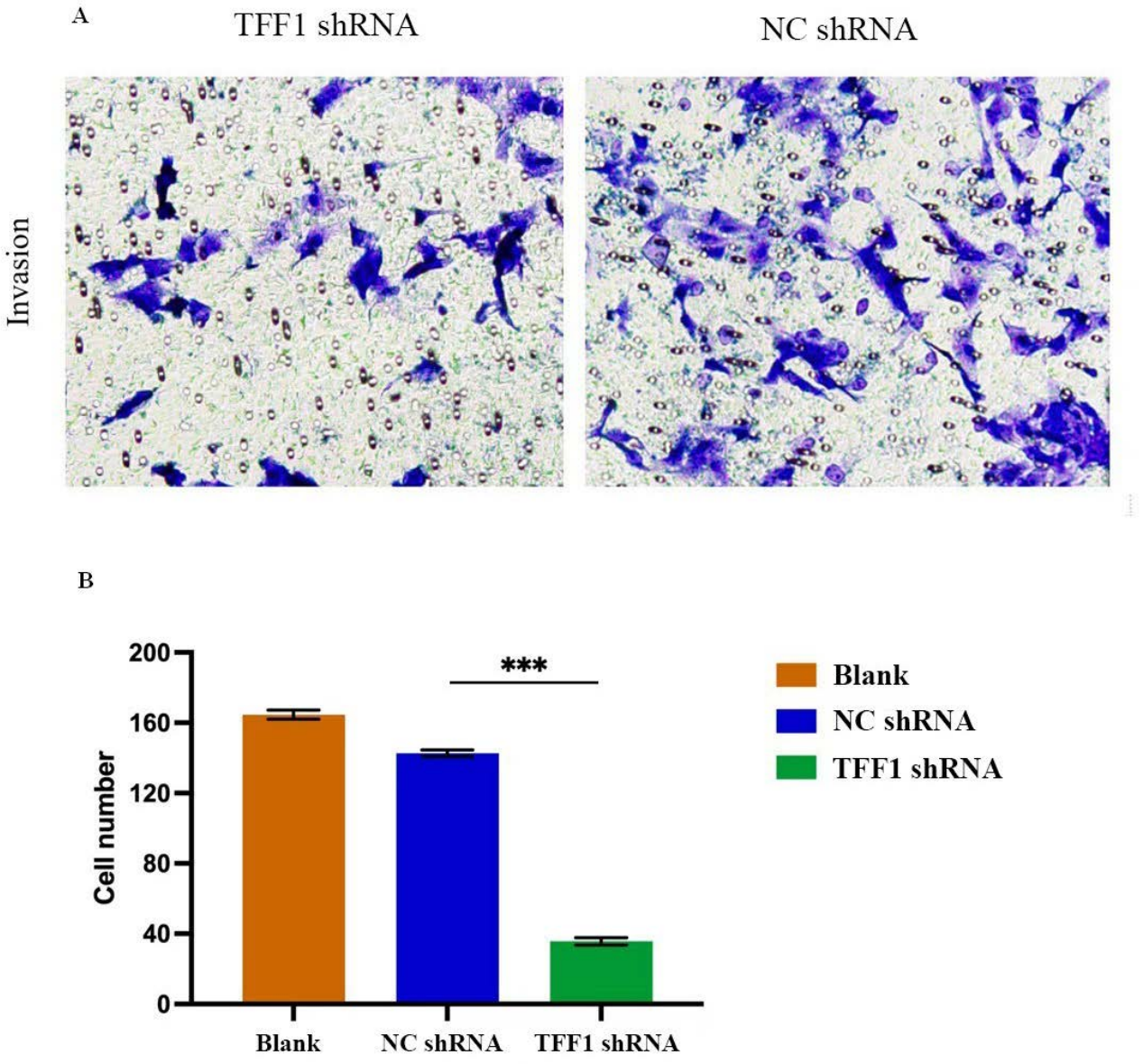


**Fig. 5 – Invasion ability analysis: A) representative images of invasive cells (crystal violet staining, magnification ×200) and B) quantitative analysis of transmembrane cell numbers.**
**For abbreviations, see Figures 1 and 3.**
**Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.**

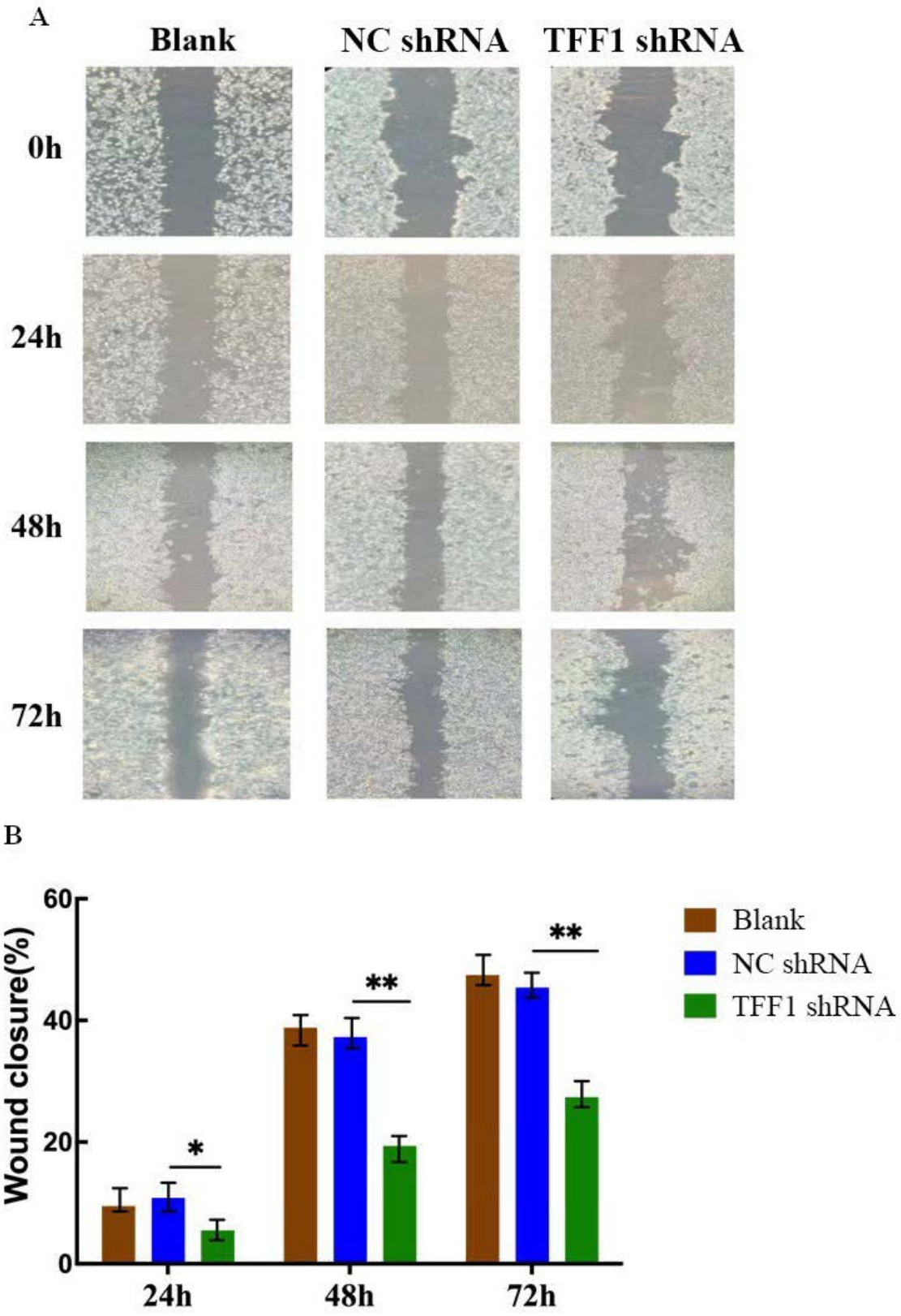


**Fig. 6 – Cell migration capability analysis: A) representative images of wound healing and B) quantitative analysis of wound closure percentage.**
**For abbreviations, see Figures 1 and 3.**
**Results are shown as mean ± standard deviation of three independent experiments. *$p < 0.05$.**

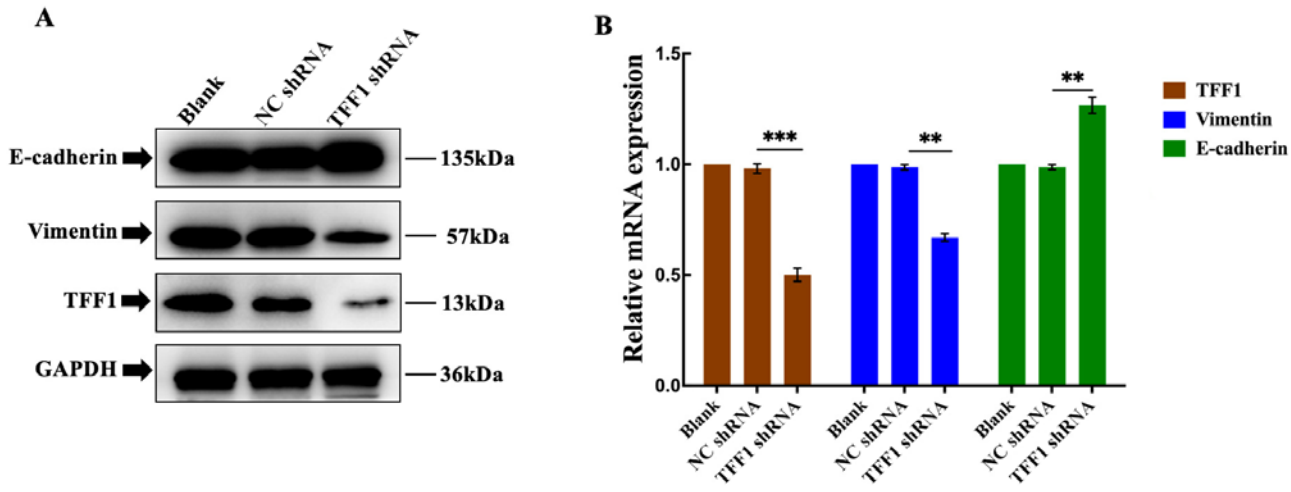


**Fig. 7 – Relationship between TFF1 expression and epithelial-mesenchymal transition: A) Western blotting analysis of E-cadherin and vimentin protein expression and B) qPCR analysis of E-cadherin and vimentin mRNA expression. For abbreviations, see Figures 2 and 3. Results are expressed as mean ± standard deviation of three independent experiments. *$p < 0.05$.**

## Discussion

The identification of aberrant genes associated with CRC and a comprehensive understanding of its pathogenic mechanisms is of paramount importance for early disease diagnosis and the identification of potential therapeutic targets. The initiation and progression of CRC is a complex process influenced by various factors, including the activation of oncogenes, the inactivation of tumor suppressor genes, and the abnormal activation of signaling pathways. At present, well-defined genes in this context encompass *RAS*, *p53*, *APC*, dMMR, and so on [15–20].

The TFF family consists of a group of small polypeptide compounds, primarily secreted by gastrointestinal mucosal epithelial cells. The mammalian TFF family comprises three members, namely TFF1 (commonly known as breast cancer-associated peptide or pS2), TFF2 (spasmolysin or SP), and TFF3 (intestinal trefoil factor or ITF) [4, 5, 21, 22]. All these proteins share a common structural feature, the "clover" domain (P domain) [23].

As a critical member of the TFF family, *TFF1* is located on chromosome 21q, and its gene structure encompasses three exons, two introns, and two transcription promoters [24]. Presently, there is limited research concerning the influence of *TFF1* on the proliferation, invasion, and migration of CRC, as well as the underlying mechanisms.

In this study, immunohistochemical methods were used to assess 40 CRC tissue samples at the protein level, revealing an expression rate of TFF1 in CRC tissues amounting to 35% (14 out of 40), with 26 cases being negative, 12 being weak positive, 1 being positive, and 1 being strong positive. The 35% expression rate for TFF1 in this study is lower than 89% and 51% reported in the other two studies by the same method [6, 25]. This suggests that not all CRC cell lines exhibit up-regulation of *TFF1* gene expression, and the expression rate varies among populations in different regions.

Furthermore, Yusufu et al. [26] documented that the *TFF3* gene exhibits the potential to promote the malignant biological characteristics of CRC, including proliferation, migration, and invasion, by facilitating EMT. However, the relationship and underlying mechanism concerning TFF1 and the malignant biological behavior of CRC were not investigated in their study. Bossenmeyer-Pourié et al. [27] reported that TFF1 can lower cell proliferation by delaying G1-S cell phase transition in HCT116. TFF1 acts as a tumor suppressor in lung and gastric cancers, whereas it acts as a tumor promoter in CRC, ovarian cancer, and pancreatic cancer [28]. Therefore, it can exhibit a dual antiproliferative and antiapoptotic role [9, 27, 28]. In this study, two siRNAs were designed based on the coding sequence of TFF1, and the results indicated that siRNA2 had a stronger silencing effect on *TFF1* than siRNA1. Therefore, siRNA2 was incorporated into the lentivirus for functional evaluation in CRC cells. The functional assays unveiled that *TFF1* silencing effectively restrained the proliferation, colony-forming ability, invasion, and migration of CRC cells.

The process of EMT encompasses the conversion of tumor cells from an epithelial to a mesenchymal phenotype, which is regarded as a pivotal step in tumor cell invasion and dissemination [29–31]. This transition is characterized by the downregulation of epithelial markers and the upregulation of mesenchymal-related proteins. Notable markers of EMT include the downregulation of E-cadherin and the upregulation of N-cadherin or vimentin. The outcomes of this study revealed that TFF1 silencing exerts an inhibitory impact on EMT. In light of previous research by Yusufu et al. [26], which suggested that the *TFF3* gene enhances CRC malignancy by promoting EMT, it is plausible to hypothesize

that the *TFF1* gene may similarly contribute to CRC malignancy by mediating EMT.

Although some studies have investigated the TFF1 protein expression in human CRC [6–10], our study is the first to use RNA interference to silence *TFF1*, revealing that silencing *TFF1* can inhibit the proliferation, invasion, and migration of human CRC cells by suppressing EMT. The obtained results indicate that TFF1 may play a significant role in the tumorigenesis and development of human CRC, which further implicates that TFF1 can be regarded as a potential marker for poor prognosis in human CRC.

## Conclusion

In summary, the findings indicate that silencing trefoil factor 1 can effectively suppress the proliferation, invasion, and migration of colorectal cancer Caco-2 cells, thereby suggesting that trefoil factor 1 may play a significant role in the initiation, progression, and metastasis of colorectal cancer by facilitating epithelial-mesenchymal transition.

## Conflict of interest

The authors declare no conflict of interest.

## Funding

This study was supported by Grants from the Tianjin Natural Science Foundation (21JCYBJC01730) and the Youth Foundation of the Second Hospital of Tianjin Medical University (Grant No. 2019ydey02, 2020ydey20), which paid all editing and publication fees.

## R E F E R E N C E S

1. *Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A,* et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021; 71(3): 209–49.
2. *Baidoun F, Elshiwy K, Elkeraie Y, Merjaneh Z, Khoudari G, Sarmini MT,* et al. Colorectal Cancer Epidemiology: Recent Trends and Impact on Outcomes. Curr Drug Targets 2021; 22(9): 998–1009.
3. *Klimeck L, Heisser T, Hoffmeister M, Brenner H.* Colorectal cancer: A health and economic problem. Best Pract Res Clin Gastroenterol 2023; 66: 101839.
4. *Hoffmann W.* Trefoil Factor Family (TFF) Peptides and their Different Roles in the Mucosal Innate Immune Defense and More: An Update. Curr Med Chem 2021; 28(36): 7387–99.
5. *Hoffmann W.* Trefoil Factor Family (TFF) Peptides and Their Diverse Molecular Functions in Mucus Barrier Protection and More: Changing the Paradigm. Int J Mol Sci 2020; 21(12): 4535.
6. *Huang YG, Li YF, Pan BL, Wang LP, Zhang Y, Lee WH,* et al. Trefoil factor 1 gene alterations and expression in colorectal carcinomas. Tumori 2013; 99(6): 702–7.
7. *Yusup A, Huji B, Fang C, Wang F, Dadihan T, Wang HJ,* et al. Expression of trefoil factors and TWIST1 in colorectal cancer and their correlation with metastatic potential and prognosis. World J Gastroenterol 2017; 23(1): 110–20.
8. *Lutz F, Han SY, Büyücek S, Möller K, Viehweger F, Schlichter R,* et al. Expression of Trefoil Factor 1 (TFF1) in Cancer: A Tissue Microarray Study Involving 18,878 Tumors. Diagnostics (Basel) 2024; 14(19): 2157.
9. *Vizoso FJ, Fagilde MC, Corte MD, Corte MG, Gava R, Bongera M,* et al. Cytosolic levels of an estrogen-induced breast cancer-associated peptide (TFF1/pS2) in colorectal cancer: Clinical significance and relationship with steroid receptors. Int J Biol Markers 2003; 18(4): 301–10.
10. *Junco A, Rodríguez JC, Allende MT, García-Muñiz JL, González JJ, Vizoso F.* The pS2 protein in primary colorectal carcinomas and in surrounding mucosa. Int J Biol Markers 1999; 14(3): 189–91.
11. *Spadazzi C, Mercatali L, Esposito M, Wei Y, Liverani C, De Vita A,* et al. Trefoil factor-1 upregulation in estrogen-receptor positive breast cancer correlates with an increased risk of bone metastasis. Bone 2021; 144: 115775.
12. *Sunagawa M, Yamaguchi J, Kokuryo T, Ebata T, Yokoyama Y, Sugawara G,* et al. Trefoil factor family 1 expression in the invasion front is a poor prognostic factor associated with lymph node metastasis in pancreatic cancer. Pancreatology 2017; 17(5): 782–7.
13. *Matsubara D, Yoshimoto T, Soda M, Amano Y, Kihara A, Funaki T,* et al. Reciprocal expression of trefoil factor-1 and thyroid transcription factor-1 in lung adenocarcinomas. Cancer Sci 2020; 111(6): 2183–95.
14. *Kunze E, Krassenkova I, Fayyazi A.* Tumor-associated neoexpression of the pS2 peptide and MUC5AC mucin in primary adenocarcinomas and signet ring cell carcinomas of the urinary bladder. Histol Histopathol 2008; 23(5): 539–48.
15. *Afrăsânie VA, Marinca MV, Alexa-Stratulat T, Gafton B, Păduraru M, Adavidoaiei AM,* et al. KRAS, NRAS, BRAF, HER2 and microsatellite instability in metastatic colorectal cancer-practical implications for the clinician. Radiol Oncol 2019; 53(3): 265–74.
16. *Müller MF, Ibrahim AE, Arends MJ.* Molecular pathological classification of colorectal cancer. Virchows Arch 2016; 469(2): 125–34.
17. *Bhullar DS, Barriuso J, Mullamitha S, Saunders MP, O'Dwyer ST, Aziz O.* Biomarker concordance between primary colorectal cancer and its metastases. EBioMedicine 2019; 40: 363–74.
18. *Harada S, Morlote D.* Molecular Pathology of Colorectal Cancer. Adv Anat Pathol 2020; 27(1): 20–6.
19. *Aghabozorgi AS, Bahreyni A, Soleimani A, Bahrami A, Khazaei M, Ferns GA,* et al. Role of adenomatous polyposis coli (APC) gene mutations in the pathogenesis of colorectal cancer; current status and perspectives. Biochimie 2019; 157: 64–71.
20. *Huang D, Sun W, Zhou Y, Li P, Chen F, Chen H,* et al. Mutations of key driver genes in colorectal cancer progression and metastasis. Cancer Metastasis Rev 2018; 37(1): 173–87.
21. *Hoffmann W.* Trefoil Factor Family (TFF) Peptides and Their Links to Inflammation: A Re-evaluation and New Medical Perspectives. Int J Mol Sci 2021; 22(9): 4909.
22. *Braga Emidio N, Hoffmann W, Brierley SM, Muttenthaler M.* Trefoil Factor Family: Unresolved Questions and Clinical Perspectives. Trends Biochem Sci 2019; 44(5): 387–90.
23. *Wright NA, Hoffmann W, Otto WR, Rio MC, Thim L.* Rolling in the clover: trefoil factor family (TFF)-domain peptides, cell migration and cancer. FEBS Lett 1997; 408(2): 121–3.
24. *Tomasetto C, Rockel N, Mattei MG, Fujita R, Rio MC.* The gene encoding the human spasmolytic protein (SML1/hSP) is in 21q 22.3, physically linked to the homologous breast cancer marker gene BCEI/pS2. Genomics 1992; 13(4): 1328–30.

25. *Welter C, Theisinger B, Rio MC, Seitz G, Schüder G, Blin N.* Expression pattern of breast-cancer-associated protein pS2/BCEI in colorectal tumors. Int J Cancer 1994; 56(1): 52–5.
26. *Yusufu A, Shayimu P, Tuerdi R, Fang C, Wang F, Wang H.* TFF3 and TFF1 expression levels are elevated in colorectal cancer and promote the malignant behavior of colon cancer by activating the EMT process. Int J Oncol 2019; 55(4): 789–804.
27. *Bossenmeyer-Pourié C, Kannan R, Ribieras S, Wendling C, Stoll I, Thim L,* et al. The trefoil factor 1 participates in gastrointestinal cell differentiation by delaying G1-S phase transition and reducing apoptosis. J Cell Biol 2002; 157(5): 761–70.
28. *Minegishi K, Dobashi Y, Tsubochi H, Hagiwara K, Ishibashi Y, Nomura S,* et al. TFF-1 Functions to Suppress Multiple Phenotypes Associated with Lung Cancer Progression. Onco Targets Ther 2021; 14: 4761–77.
29. *Mittal V.* Epithelial Mesenchymal Transition in Tumor Metastasis. Annu Rev Pathol Mech Dis 2018; 13: 395–412.
30. *Manfioletti G, Fedele M.* Epithelial-Mesenchymal Transition (EMT) 2021. Int J Mol Sci 2022; 23(10): 5848.
31. *Pastushenko I, Blanpain C.* EMT Transition States during Tumor Progression and Metastasis. Trends Cell Biol 2019; 29(3): 212–26.

Received on July 16, 2025
Revised on August 8, 2025
Revised on September 23, 2025
Accepted on October 8, 2025
Online First November 2025