

77566_Auto_Edited-check.docx

Name of Journal: *World Journal of Gastrointestinal Oncology*

Manuscript NO: 77566

Manuscript Type: ORIGINAL ARTICLE

Basic Study

Transcriptional factor III A promotes colorectal cancer progression by upregulating cystatin A

Wang J *et al.* GTF3A promotes CRC progression

Abstract

BACKGROUND

Advanced colorectal cancer (CRC) generally has poor outcomes and high mortality rates. Clarifying the molecular mechanisms underlying CRC progression is necessary to develop new diagnostic and therapeutic strategies to improve CRC outcome and decrease mortality. Transcriptional factor III A (GTF3A), an RNA polymerase III transcriptional factor, is a critical driver of tumorigenesis, and aggravates CRC cell growth.

AIM

To confirm whether GTF3A promotes CRC progression by regulating the expression of cystatin A (CSTA) gene and investigate whether GTF3A serves as a prognostic biomarker and therapeutic target for patients with CRC.

METHODS

Human tissue microarrays containing 90 pairs of CRC tissues and adjacent non-tumor tissues, and human tissue microarrays containing 20 pairs of CRC tissues, adjacent non-tumor tissues, and metastatic tissues were examined for GTF3A expression using immunohistochemistry. The survival rates of patients were analyzed. Short hairpin GTF3As and CSTAs were designed and packaged into the virus to block the expression of *Gtf3a* and *Csta* genes, respectively. *In vivo* tumor growth assays were performed to confirm whether GTF3A promotes CRC cell proliferation *in vivo*. Electrophoretic mobility shift assay and fluorescence *in situ* hybridization assay were used to detect the interaction of GTF3A with *Csta*, whereas luciferase activity assay was used to evaluate the expression of *Gtf3a* and *Csta* gene. RNA-Sequencing (RNA-Seq) and data analyses were used to screen for target genes of GTF3A.

RESULTS

The expression of GTF3A was higher in CRC tissues and lymph node metastatic tissues than in the adjacent normal tissues. GTF3A was associated with CRC prognosis, and knockdown of the *Gtf3a* gene impaired CRC cell proliferation, invasion and motility *in vitro* and *in vivo*. Moreover, RNA-Seq analysis revealed that GTF3A might upregulate the expression of *Csta*, whereas the luciferase activity assay showed that GTF3A bound to the promoter of *Csta* gene and increased *Csta* transcription. Furthermore, CSTA regulated the expression of epithelial-mesenchymal transition (EMT) markers.

CONCLUSION

GTF3A increases CSTA expression by binding to the *Csta* promoter, and increased CSTA levels promotes CRC progression by regulating EMT. Inhibition of GTF3A prevents CRC progression. Therefore, GTF3A is a potential novel therapeutic target and biomarker for CRC.

Key Words: Transcription factor IIIA; Cystatin A; Colorectal cancer

Wang J, Tan Y, Jia QY, Tang FQ. Transcriptional factor III A promotes colorectal cancer progression by upregulating cystatin A. *World J Gastrointest Oncol* 2022; In press

Core Tip: Transcriptional factor III A (GTF3A) is highly expressed in colorectal cancer (CRC) tissues, and GTF3A expression is associated with CRC prognosis. GTF3A binds to the promoter of cystatin (CSTA) gene to facilitate *Csta* transcription, which regulates the expression of epithelial-mesenchymal transition markers and promotes CRC progression. Blocking GTF3A significantly inhibits CRC cell growth. Therefore, GTF3A is a potential novel therapeutic target and prognostic biomarker for CRC.

2

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the leading cause of cancer-related death worldwide^[1]. CRC has distant invasive and metastatic

abilities, such as liver and lung metastases, resulting in poor survival^[2]. Clarifying the molecular mechanisms underlying CRC progression is necessary for the development of new therapeutic strategies. Transcriptional factor III A (GTF3A), an RNA polymerase III transcriptional factor, specifically binds to the internal control region of the 5S rRNA gene from nucleotides +43 to +96^[3], initiating the assembly of the transcription initiation complex (5S rDNA-TFIIIA-TFIIIC2-TFIIIBbeta complex). Complex formation is not proportional to the amount of TFIIIA^[4]. GTF3A is present in all organism of human. GTF3A shares a common conserved transcription activating signal, nuclear localization signal, and nuclear export signal, but lacks initiated Met and accompanying conserved residues in the N-terminal regions^[5]. GTF3A regulates the 5S rRNA synthesis network by binding to 5S rDNA and 5S rRNA^[6]. GTF3A binds to 5S rRNA to form the 7S ribonucleoprotein particle (RNP) complex, and the complex functions as an NES to transfer 5S rRNA to the cytoplasm depending on the NES sequence, which protects the 5S rRNA from degradation^[7,8]. Several studies have suggested that 5S rRNA binds to L5 and L11 to form the 5S RNP complex, regulating the MDM2-p53 checkpoint^[9-12]. Alterations in ribosome biogenesis are critical drivers of tumorigenesis, and are closely associated with increased CRC cell growth^[13]. These findings suggest that GTF3A regulates CRC progression.

Cystatin A (CSTA), a cysteine proteinase inhibitor, is a type 1 cystatin (stefin). CSTA is a cornified cell envelope constituent of keratinocytes that plays a critical role in epidermal development and maintenance. The high expression of CSTA is associated with the invasion and metastasis of various malignant tumors, such as pancreatic ductal adenocarcinoma^[14], esophageal squamous cell carcinoma^[15], lung cancer^[16], hepatocellular carcinoma (HCC)^[17], and nasopharyngeal carcinoma (NPC)^[18]. An increasing number of studies have shown that CSTA is a potential prognostic and diagnostic biomarker for cancer progression. The activity of the CSTA promoter is positively regulated by the active Ras/MEKK1/MKK7/JNK signal transduction pathway, but negatively regulated by the negative Ras/Raf-1/MEK1/ERK pathway in human keratinocytes^[19]. Other cysteine protease inhibitors, cystatin SN (CST1) and

cystatin S (CST4) are type 2 cystatin proteins, which enhance the metastasis of various malignant tumors and contribute to poor patient survival^[20,21]. CST1 overexpression increases cell migration and invasion through mediating the epithelial-mesenchymal transition (EMT) in breast cancer and HCC^[22,23] and contributes to CRC cell proliferation^[24]. CST1 is also considered an early diagnostic biomarker and potential therapeutic target in breast, CRC, and gastric cancers^[25,26]. In the present study, we showed that GTF3A was highly expressed in CRC, and it bound to the promoter of *Csta* to facilitate *Csta* transcription, which then regulated EMT marker expression and promoted CRC progression. Therefore, GTF3A is a potential novel therapeutic target and prognostic biomarker for CRC.

MATERIALS AND METHODS

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), Cell Counting Kit (CCK8), and other supplements were obtained from Life Technologies (Rockville, MD, United States). GTF3A antibody for western blotting was purchased from Bethyl Laboratories, Inc (Suzhou, China). CSTA antibody was purchased from Novus (CO, United States). CST1 antibody was purchased from Invitrogen (Shanghai, China). CST4 antibody was purchased from R&D Systems (Minneapolis, MN, United States). GTF3A antibody used for the immunofluorescence assay was purchased from Bioss Antibodies (Beijing, China). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI, United States). Antibodies against Snail, E-cadherin, and beta-catenin were purchased from Abcam (Cambridge, United Kingdom). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and secondary antibodies were purchased from Proteintech Company (Wuhan, Hubei, China).

Tissue microarray and immunohistochemical staining

Human tissue microarrays (HCol-Ade180Sur-08) containing 90 pairs of CRC tissues and adjacent non-tumor tissues, and human tissue microarrays (HCol-Ade060Lym-01)

containing 20 pairs of CRC tissues, adjacent non-tumor tissues, and lymph node metastatic tissues, were purchased from Outdo Biotech Company (Shanghai, China). Immunohistochemical (IHC) staining was performed to detect the expression of GTF3A as described previously^[27]. These tissue microarrays were stained with GTF3A antibody (1:300 dilution). The use of patient materials was approved by the ethics committee of Hunan Cancer Hospital (No:KYJJ-2020-004). All IHC results were evaluated based on the semi-quantitative histological scoring (HSCORE) system using the following formula: $H\text{-SCORE} = \sum (p_i \times i)$ [p_i represents the percentage of stained cells in an intensity area, whereas i represents the staining intensity (0, no labeling; 1, weak; 2, moderate; and 3, strong)].

Cell lines

Human CRC cell lines (HCT116, SW480, DLD1, SW620, and HT29) were obtained from the American Type Culture Collection (Manassas, VA, United States) and were purchased from the Shanghai Cell Center (Shanghai, China). All cell lines were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C and 5% CO₂.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed as described previously^[28]. Briefly, the total RNA of cultured cells was extracted using a total RNA kit (R6834, OMEGA), and 1 µg of DNase-treated RNA was reverse transcribed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's instructions. The threshold cycle (Ct) values were measured using Hieff quantitative PCR SYBR Green Mix (Yeasen, Shanghai, China) in A LightCycler 96 qPCR System (Roche). Primer sequences used are listed in Supplementary Table 1. The relative mRNA levels of each gene were normalized to those of the housekeeping gene GAPDH. Relative transcript levels were

calculated as two power values of ΔC_t (the differential value of C_t between GAPDH and the target cDNA).

Plasmid transfection

The designed short hairpin RNAs (shRNAs), shGTF3A#1, shGTF3A#2, shGTF3A#3, shGTF3A#4, and shscramble were cloned into the lentivirus vector GV112 by Shanghai GeneKai Company (Shanghai, China). The shRNA sequences for *Gtf3a* are listed in Supplementary Table 2. The shRNAs for *Csta* gene were designed to knockdown the expression of CSTA. The shRNA sequences for *Csta* are shown in Supplementary Table 3. Synthetic plasmids were verified by sequence analysis and PCR and then cloned into GV493. Lipofectamine 3000 (Thermo Fisher Scientific, Carlsbad, CA, United States) was used to transfect the plasmids into HEK293T cells; 1×10^5 cells were transfected with shscramble, shGTF3A#1, shGTF3A#2, shGTF3A#3, shGTF3A#4, shCSTA#1, and shCSTA#2. The knockdown efficiency was filtered using RT-qPCR.

Stable cell lines

shGTF3As, shCSTAs and negative control lentiviruses were packaged by the Shanghai GeneKai Company (Shanghai, China). The lentivirus titers were quantified ($\geq 10^8$ TU/mL). Following the manufacturer's protocol, 2×10^5 of SW480 and HCT116 cells were seeded in a six-well culture plate, and cultivated at 37 °C and 5% CO₂. After 24 h, the cells were transfected with the appropriate amount of lentivirus at multiplicity of infection of 20 TU/mL. After being incubated for 10 h, the culture medium was removed, and fresh 10% FBS DMEM was added. Next, antibiotic-free medium containing 3 µg/mL puromycin was used to screen the stable cells for 3-4 wk. Ultimately, the knockdown and negative control stable cell lines, shscramble-SW480, shGTF3A#1-SW480, shGTF3A#4-SW480, shGTF3A#1-HCT116, and shGTF3A#4-HCT116 were obtained.

Western blotting

Western blotting was performed as previously described^[29]. Briefly, 1×10^6 cells were lysed with a radioimmunoprecipitation assay lysis buffer [50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing $100 \times$ protease inhibitor cocktail and $100 \times$ phosphatase inhibitor cocktail (CWBIO, Beijing, China). Following the manufacturer's instructions, the crude lysate was centrifuged and the supernatant was collected to measure the protein concentration using the BCA Protein Assay Kit (CWBIO, Beijing, China). After being boiled at 100 °C for 5 min, 20-60 µg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to the 0.2 µm PVDF membrane (Millipore). The protein membrane was blocked with 5% non-fat milk, incubated with the indicated primary antibody, and incubated with an appropriate peroxidase conjugated secondary antibody. The signal was detected on a gel imager using an enhanced chemiluminescence (ECL) western blotting kit (CWBIO, Beijing, China). GAPDH was used as an internal control to verify basal expression. The ratio of specific proteins to GAPDH was calculated.

Cell proliferation and colony formation assays

shGTF3A#1-HCT116, shGTF3A#4-HCT116, shscramble-HCT116, shGTF3A#1-SW480, shGTF3A#4-SW480, and shscramble-SW480 cells were seeded in 96-well plates, and CCK8 (ApexBio) was used to examine cell viability following the manufacturer's protocol. Briefly, CCK8 was added to the cell plates, and incubated for 4 h. Then, the optical density at 450 nm was measured using a microplate reader. Cell viability was calculated daily for 5 d. For the colony formation assay, shGTF3A#1-HCT116, shGTF3A#4-HCT116, shscramble-HCT116, shGTF3A#1-SW480, shGTF3A#4-SW480, and shscramble-SW480 cells were seeded in 6-well plates with each well containing 1000 cells, and cultured for 16 d. The cell colonies were fixed in methanol, stained with 0.5% gentian violet, and counted automatically using a computerized microscope system.

Cell invasion and motility assay

Cell invasion and motility assays *in vitro* were performed using previously described methods^[29]. Briefly, for the invasion assay, Matrigel (25 mg/50mL, Collaborative Biomedical Products, Bedford, MA, United States) was added to the upper chamber with 8 mm pore polycarbonate membrane filters. shGTF3A#1-HCT116, shGTF3A#4-HCT116, shscramble-HCT116, shGTF3A#1-SW480, shGTF3A#4-SW480, and shscramble-SW480 cells were seeded in the upper chamber (Neuro Probe, cabin John, MD) at density of 1.5×10^4 cells/well in 100 μ L of serum-free medium, and then incubated at 37 °C for 48 h. The bottom chamber contained standard medium with 20% FBS. Cells that invaded the lower surface of the membrane were fixed with 37% paraformaldehyde, and stained with crystal violet. Invading cells were counted under a light microscope. The motility assay was performed in a similar manner to the invasion assay without Matrigel coating.

Wound healing assay

A wound healing assay was used to measure the cell migration potential. Briefly, 2×10^5 of shGTF3A#1-HCT116, shGTF3A#4-HCT116, shscramble-HCT116, shGTF3A#1-SW480, shGTF3A#4-SW480, and shscramble-SW480 cells were seeded in 6-well plates. After the cells reached 95% confluence, the surface of the cell layer was wounded using a sterile 10 μ L pipette tip. The cells were then rinsed three times with phosphate buffered saline (PBS) to move detached cells and incubated in DMEM containing 1% FBS for 48 h. Wound closure was observed under a microscope at 0, 24, and 48 h.

Tumor growth assays in vivo

In vivo tumor growth assays were performed as previously described^[30]. Briefly, female nude mice (aged 4-5 wk) were obtained from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). Experiments involving animal subjects and protocols for animal studies were approved by the Laboratory Animal Research Center of Hunan Cancer Hospital (No: 2020-118). Nude mice were subcutaneously injected with 3×10^6

shGTF3A#1-SW480, shGTF3A#4-SW480, or shscramble-SW480 cells (5 mice per group). The size of the tumor that developed in the mice was measured every 3 d, and a tumor growth curve was drawn. After 4 wk, the mice were euthanized with pentobarbital sodium at 20 mg/ml, and the tumor weights were measured.

RNA-Sequence and data analysis

After harvesting shGTF3A#1-SW480, shGTF3A#4-SW480, and shscramble-SW480 cells, total RNA was extracted using TRIzol reagent (Invitrogen). After being removed the rRNA, the enriched longRNA (> 200 nt) was interrupted, reversely transcribed into cDNA, repaired, and RNA-Sequencing (RNA-Seq) to build a chain-specific database. The clean data were obtained by quality testing of Bioptic Qsep100, and then comparative mapping of genomes was acquired between clean data and the hg38 Ensemble transcriptome using HISAT2 software. The differentially expressed genes between groups were analyzed using the DESeq2 R package, and the default screening criteria were: (1) Log2 (fold change) >1; and (2) False discovery rate < 0.05.

Fluorescence in situ hybridization assay

Fluorescence *in situ* hybridization (FISH) assays were performed as previously described^[31]. FISH probes for *Csta* promoter sequence were designed and synthesized by Well Bio (Guangzhou, China). The lncRNA FISH kit (C10910) was purchased from Wellbio (Guangzhou, China). The cell slides were fixed with 4% paraformaldehyde for 30 min at 25 °C, permeabilized with proteinase K for 30 min at 37 °C, and blocked for 30 min at 37 °C with 200 µL of pre-hybridization solution. After removing the pre-hybridization solution, 100 µL of the probe hybridization solution was added to the cell slides overnight at 37 °C in the dark. After being washed three times for 5 min each with 4 × SSC, 2 × SSC, 1 × SSC, and PBS, the cell slides were incubated with the appropriately diluted primary antibody (anti-GTF3A) overnight at 4 °C. After washing three times with PBS, the slides were incubated with the secondary anti-rabbit immunoglobulin G-labeled fluorescent antibody for 90 min at 37 °C. Finally, the slides

were stained with 4',6-diamidino-2-phenylidole for 10 min at 37 °C and sealed with 90% glycerin. All images were acquired using fluorescence microscope.

Electrophoretic mobility shift assay

A biotin labeled *Csta* promoter DNA probe (5'-biotin-agctagtgacgccttttaaacacgtccaccattccttccttttttc-3') was synthesized by Sangon (Shanghai, China). Both sense and antisense strands were diluted to a concentration of 0.2 μ M, mixed at 1:1, denatured at 95 °C for 5 min, maintained at 70 °C for 20 min, and formed into a double-stranded probe (diluted 10 times when used) after being cooled to room temperature. Following the instructions of the Electrophoretic mobility shift assay (EMSA) kit (CWBIO, Beijing, China), the protein supernatant for each sample was separated on a 4% polyacrylamide gel, and transferred to a nylon membrane (CWBIO). The protein membrane was cross-linked using UV radiation, blocked with a confining liquid, and incubated with the indicated streptavidin-horseradish peroxidase conjugate. The signal was detected on gel imager using ECL western blotting kit (CWBIO, Beijing, China).

Luciferase activity assay

Dual-luciferase reporter assay system (Promega) was used to detect the expression of *Csta* following the manufacturer's instructions. Briefly, the *Csta* promoter was cloned into the firefly luciferase plasmid, and *Csta* promoter-luc was obtained. Then, 1×10^5 of HCT116 cells were cultured in 48-well plates. *Csta* promoter-luc, plasmid-Renilla, and plasmid-GTF3A were co-transfected into HCT116 cells with X-tremeGene HP. After 48 h culture, the cells were harvested, lysed with Passive Lysis Buffer, and treated with Luciferase Assay Reagent. Firefly luciferase and renilla luciferase were measured. The ratio of firefly luciferase to renilla Luciferase represents the expression of *Csta*.

Statistical analysis

All experiments were performed at least three times. The results are presented as the mean \pm SD of three independent experiments and were analyzed using student's *t*-test.

The differences between groups are reported as follows: ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$; and ns: $P > 0.05$. All statistical data were calculated using GraphPad Prism software (version7.0).

RESULTS

High expression of GTF3A in CRC and metastatic tissues

To determine the expression of GTF3A in CRC tissues, two sets of CRC tissue microarray were used to detect GTF3A expression with IHC. GTF3A was found to be higher expression in the cancer tissues than that in the adjacent tissues of HCol-Ade180Sur-08 microarray. To further probe whether metastatic cancers had a higher expression and analyze the association of GTF3A expression with metastasis, HCol-Ade060Lym-01 microarray containing CRC cancer, metastatic tissues, and adjacent tissues was also used to detect GTF3A, and observe GTF3A expression in metastatic tissues. These two microarrays had total 110 cancer tissue, 110 adjacent tissues, and 20 metastatic tissues, some tissue of them were chipped off and could not be used. 102 cases in the cancer group, 106 cases in adjacent the group and 20 cases in metastatic group were calculated by gray scanning and scored, and survival time and survival curve were analyzed. ⁶ The expression of GTF3A in CRC and metastatic tissues was higher than that in adjacent normal tissues (Figures 1A and 1B, $P < 0.01$). To analyze whether GTF3A expression was relevant to survival time, patients with CRC were divided into GTF3A negative and positive groups, and the survival curve of CRC patients showed that the negative group had longer overall survival than the positive group (Figure 1C, $P < 0.05$). These clinical data suggested that GTF3A expression is associated with CRC progression.

Knockdown of the Gtf3a gene inhibits CRC cell proliferation

These results indicate that *Gtf3a* gene may promote CRC progression. Five CRC cell lines, HCT116, SW480, DLD1, SW620, and HT-29 were used to detect the expression of GTF3A using western blotting. The results showed that SW480 cells had high

expression of GTF3A, whereas HCT116, DLD1, SW620, and HT-29 cells had low expression of GTF3A (Figure 2A). To clarify the role of *Gtf3a* in CRC, shGTF3A#1, #2, #3, and #4 were designed and packaged into the virus. Their inhibitory effects on *Gtf3a* were screened, and the results showed that shGTF3A#1 and shGTF3A#4 had high knockdown efficiencies. HCT116 and SW480 cells were stably transfected with shscramble, shGTF3A#1, or shGTF3A#4. RT-qPCR was performed to evaluate knockdown efficiency, and the results showed that shGTF3A#1, and shGTF3A#4 were effectively knocked down in HCT116 and SW480 cells (Figures 2B). Consistently, Western blotting results showed that GTF3A protein expression was effectively decreased in shGTF3A#1,4-HCT116 and shGTF3A#1,4-SW480 cells (Figure 2B). The cell viability of HCT116 and SW480 cells was detected using the CCK8 assay after knockdown of the *Gtf3a* gene, and the cell proliferation of HCT116 and SW480 cells was significantly decreased in the knockdown group (Figure 2B). Furthermore, the cell colony formation assay showed that colony size and number were dramatically diminished in *Gtf3a*-knockdown cells (Figure 2C, $P < 0.01$). These data indicated that knockdown of *Gtf3a* inhibits the growth of CRC cells.

Knockdown of *Gtf3a* inhibits CRC cell motility and invasion

Gtf3a-knockdown SW480 and HCT116 cells were used to determine whether *Gtf3a* is involved in CRC cell motility and invasion. Results of the wound healing assay showed that shGTF3A#1,4-HCT116 and shGTF3A#1,4-SW480 cells had impaired migratory capability compared with shscramble cells (Figure 3A). In addition, the Transwell assay showed that invasion and metastasis in the *Gtf3a*-knockdown groups were significantly repressed compared with those in the controls (Figure 3B), and the number of invaded or migrated cells in the knockdown groups dramatically decreased (Figure 3B). Collectively, these results highlight that knockdown of *Gtf3a* suppressed CRC cell invasion and metastasis *in vitro*, whereas the *Gtf3a* gene promoted the progression of CRC.

GTF3A protein regulates CSTA by binding to the CSTA promoter

To explore the molecular mechanisms of GTF3A in CRC progression, RNA-Seq was used to screen the target genes of GTF3A, and the differential genes between SW480 *Gtf3a*-knockdown and scramble control cells were analyzed. The RNA-Seq results are shown in Figures 4A and 4B; several differentially expressed genes were found between the groups (Figures 4A and 4B, Supplementary Table 4). In particular, the number of *Csta/Cst1/Cst4* genes in *Gtf3a*-knockdown cells was dramatically lower than that in control group (Supplementary Table 4). *Csta/Cst1/Cst4* genes, members of the cystatin superfamily that encode cysteine protease inhibitors, are overexpressed in various types of cancers, subsequently promoting cancer cell metastasis and resulting in poor prognosis^[14-16]. RT-qPCR and western blotting results showed that the number of *Csta/Cst1/Cst4* genes was significantly decreased in *Gtf3a*-knockdown cells (Figures 4C and 4D).

The results of the RNA-Seq showed that CSTA had the largest difference after knockdown of *Gtf3a* in FISH experiments, the sequence probe of the *Csta* promoter was labeled with red fluorescence, whereas GTF3A was labeled with green fluorescence. The fluorescence staining of the GTF3A and *Csta* promoters was colocalized to a large extent as indicated by an orange-yellow fused fluorescence (Figure 4E), suggesting that GTF3A combined with the *Csta* promoter. To confirm the interaction between GTF3A and the *Csta* promoter, EMSA was performed to directly observe the interaction GTF3A and the *Csta* promoter. The results showed that GTF3A interacted with the *Csta* promoter (Figure 4F). Next, a dual-luciferase assay was carried out to determine whether the interaction of GTF3A with *Csta* promoter increased *Csta* expression, and the results showed that the transcript activity of the *Csta* gene was significantly increased after transfection with CSTA (Figure 4G, $P < 0.05$). These data suggested that GTF3A binds to the *Csta* promoter to regulate its transcription and translation.

GTF3A mediates CRC cell EMT by through regulating the expression of CSTA

The results indicated that GTF3A promoted CRC cell invasion and metastasis. To investigate the underlying mechanisms, EMT markers, such as Snail, E-cadherin, and beta-catenin were detected in scrambled control and *Gtf3a*-knockdown cells. Western blotting results showed that *Gtf3a*-knockdown HCT116 and SW480 cells decreased Snail expression and increased E-cadherin expression compared with the scrambled control (Figures 5A and 5B). Moreover, GTF3A regulates the expression of CSTA/CST1/CST4, and CST1 promotes the migration and invasion of breast cancer cells by up-regulating E-cadherin^[23]. Consistent with these results, *Csta*-knockdown cells were constructed (Figure 5C), and Snail, E-cadherin, and beta-catenin were detected in these cells. The cells showed down-regulated Snail expression and upregulated E-cadherin expression (Figure 5D). Collectively, GTF3A may mediate EMT to promote CRC cell metastasis by regulating CSTA and CST1 expression.

GTF3A promotes CRC cell growth in vivo

The results showed that GTF3A promoted the proliferation, invasion, and metastasis of CRC cells *in vitro*. To verify whether GTF3A promotes CRC progression *in vivo*, nude mice were subcutaneously injected with the *Gtf3a*-knockdown and control cells to examine the function of *Gtf3a*. After 28 d, *Gtf3a*-knockdown group had a significantly smaller tumor size (Figures 6A and 6B) and slower tumor growth (Figure 6C, $P < 0.05$) than the control group. In addition, the *Gtf3a*-knockdown group showed a reduction in tumor weights (Figure 6D, $P < 0.05$). *In vivo* experiments demonstrated that GTF3A promoted the growth of CRC cells.

DISCUSSION

As a transcription factor of RNA polymerase III, GTF3A is homologous to TFIIIB and TFIIIC, which guides the accurate transcription of 5S RNA genes^[32]. RNA polymerase III is responsible for the transcription of non-coding genes including U6 snRNA, tRNA and 5S RNA^[33]. Deregulation of RNA polymerase III leads to the development of a large variety of human disorders^[34], and upregulation of RNA polymerase III

transcription has been observed in various types of cancer^[35,36]. TFIIIB and TFIIIC can enhance the transcriptional activity of RNA polymerase III and mediate cellular transformation and tumor formation^[37-40]. In addition, TFIIIA-mediated 5S rRNA is an essential component of 5S RNP that regulates the Hdm²-p53 checkpoint to affect ribosome biosynthesis and cancer progression^[9-13], indicating that GTF3A participates in the occurrence and development of various cancer types. Overexpression of GTF3A has been observed in CRC tumors and metastatic tissues using CRC tissue arrays, and clinical data analysis suggests that GTF3A is associated with CRC progression and metastasis.

⁴ A series of *in vitro* and *in vivo* experiments was performed to examine the role of GTF3A in CRC development. The results showed that the knockdown of *Gtf3a* inhibited the proliferation, invasion and metastasis of CRC cells. Generally, cancer metastasis is mostly related to EMT^[41]. We hypothesized that GTF3A promotes CRC cell metastasis by mediating the EMT. Thus, EMT biomarkers, Snail, vimentin, beta-Catenin, and E-cadherin were detected. These changes in Snail and E-cadherin levels were in accordance with our hypothesis. The vimentin had no changed in knockdown GTF3A (not shown). Both RNA-Seq and RT-qPCR showed that *Csta* expression was dramatically decreased in *Gtf3a*-knockdown cells. Furthermore, the luciferase activity assay suggested that GTF3A regulates the *Csta* transcription and translation by binding to the *Csta* promoter, therefore, *Csta* is a target gene of GTF3A.

CSTA is associated with invasion and metastasis in various cancer types^[17,19,22], and *in vitro* experiments have shown that CSTA modulates the invasion and metastasis of NPC cells^[18]. Based on our data, GTF3A may regulate *Csta* expression to mediate EMT and promote CRC metastasis. FISH and EMSA results suggested that GTF3A combined with the promoter of the *Csta* gene, and the luciferase activity assay showed that GTF3A upregulated *Csta* transcription by binding to the *Csta* promoter. In addition, CSTA regulates E-cadherin and Snail expression, there mediating the EMT shift. In conclusion, GTF3A upregulates CSTA expression to promote CRC metastasis by accelerating EMT shift. CST1 is associated with the progression and prognosis of various cancer

types^[20,25], and its overexpression modulates EMT progression by modulating the PI3K/AKT pathway *in vivo* and *in vitro*^[22].

CONCLUSION

GTF3A increased *Csta* gene transcription and protein expression by binding to the *Csta* promoter, increased in the expression of CSTA, enhanced the EMT process and facilitated CRC cell invasion and metastasis. However, knockdown of *Gtf3a* decreased CSTA expression, inhibited the EMT, and suppressed CRC cell invasion and metastasis (Figure 6E). Therefore, GTF3A is a potential novel therapeutic target and prognostic biomarker in human CRC.

ARTICLE HIGHLIGHTS

Research background

Advanced colorectal cancer (CRC) generally has poor outcomes and high mortality rates. Clarifying the molecular mechanisms underlying CRC progression is necessary to develop new diagnostic and therapeutic strategies to improve CRC outcome and decrease mortality.

Research motivation

Transcriptional factor III A (GTF3A), an RNA polymerase III transcriptional factor, is a critical driver of tumorigenesis, and aggravates CRC cell growth. The mechanism of GTF3A participating CRC is not clear.

Research objectives

To confirm whether GTF3A aggravates CRC progression and investigate molecular mechanisms underlying CRC progression.

Research methods

Immunohistochemistry was used to detect GTF3A expression CRC tissues. Short hairpin GTF3As and CSTAs were designed and packaged into the virus to block the expression of *Gtf3a* and *Csta* genes. RNA-Sequence and data analysis was used to screen the target genes of GTF3A. Fluorescence *in situ* hybridization assay were used to detect the interaction of GTF3A with *Csta*, **and** luciferase activity assay was used to evaluate the expression of *Gtf3a* and *Csta* gene.

Research results

GTF3A⁸ highly expressed in CRC tissues and metastatic tissues, its expression was associated with CRC prognosis. Knockdown of the *Gtf3a* gene impaired CRC cell proliferation, invasion and motility *in vitro* and *in vivo*. GTF3A increased *Csta* transcription, and increased CSTA upregulated epithelial-mesenchymal transition (EMT) markers.

Research conclusions

GTF3A increases CSTA expression by binding to the *Csta* promoter, and increased CSTA levels promotes CRC progression by regulating EMT. Inhibition of GTF3A prevents CRC progression.

Research perspectives

GTF3A may be a potential novel therapeutic target and biomarker for CRC.

3%

SIMILARITY INDEX

PRIMARY SOURCES

- 1

datasheets.scbt.com
Internet

33 words — 1%

- 2

Pasquale Spinelli, Giuseppe Calarco, Andrea Mancini, Xiao - Guang Ni. "Operative colonoscopy in cancer patients", Minimally Invasive Therapy & Allied Technologies, 2009
Crossref

20 words — < 1%

- 3

Nagaoka, M.. "Significant Effect of Linker Sequence on DNA Recognition by Multi-Zinc Finger Protein", Biochemical and Biophysical Research Communications, 20010413
Crossref

13 words — < 1%

- 4

Nan Wang, Jia-Xing He, Guo-Zhan Jia, Ke Wang, Shuai Zhou, Tao Wu, Xian-Li He. "The lncRNA XIST promotes colorectal cancer cell growth through regulating the miR-497-5p/FOXK1 axis", Research Square Platform LLC, 2020
Crossref Posted Content

13 words — < 1%

- 5

Kesong Peng, Guoqiang Su, Jinmeng Ji, Xiaojia Yang, Mengmeng Miao, Pingli Mo, Ming Li, Jianming Xu, Wengang Li, Chundong Yu. "Histone demethylase JMJD1A promotes colorectal cancer growth and metastasis by enhancing Wnt/ β -catenin signaling", Journal of Biological Chemistry, 2018
Crossref

12 words — < 1%

6

Meilin Weng, Hao Zhang, Wenting Hou, Zhirong Sun, Jing Zhong, Changhong Miao. "<p>ACAT2 Promotes Cell Proliferation and Associates with Malignant Progression in Colorectal Cancer</p>", OncoTargets and Therapy, 2020

Crossref

12 words — < 1%

7

Qiu, Yumin, Rongfa Yuan, Shouhua Zhang, Leifeng Chen, Da Huang, Haibin Hao, and Jianghua Shao. "Rock2 stabilizes β-catenin to promote tumor invasion and metastasis in colorectal cancer", Biochemical and Biophysical Research Communications, 2015.

Crossref

12 words — < 1%

8

thno.org

Internet

12 words — < 1%