Overexpression of long non-coding RNA-CTD903 inhibits colorectal cancer invasion and migration by repressing Wnt/β-catenin signaling and predicts favorable prognosis

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Abstract. Accumulating evidence reveals that long non-coding RNA (lncRNA) is essential for tumorigenesis and progression, but little is known about its roles and mechanisms in metastatic colorectal cancer (CRC). This study aimed to detect expression level and prognostic role of lncRNA-CTD903 in CRC patients, which was selected based on one microarray data. The effects on cell invasion, migration and proliferation were investigated after silencing or overexpression of CTD903 in CRC cell lines. We also observed the EMT (epithelial-mesenchymal transition) phenomenon and effect on cell adhesion. The associations between CTD903 and EMT markers, such as E-cadherin, N-cadherin, β-catenin, ZEB1, ZO-1, Snail, and Twist, were determined by western blotting. Our results showed lncRNA-CTD903 expression was strongly upregulated in 115 CRC patients, comparing to adjacent normal tissues. CTD903 was proven to be an independent predicted factor of favorable prognosis in CRC patients by using multivariate Cox proportional hazards model. After knockdown of CTD903 in RKO and SW480, both cell invasion and migration increased, and cells exhibited EMT-like appearance, along with reduced adhering ability. Moreover, overexpression of CTD903 in DLD1 and HCT116 reversed these phenotypes. Furthermore, downregulation of CTD903 enhanced Wnt/β-catenin activation and subsequently increased transcription factors (Twist and Snail) expression, along with increased mesenchymal marker Vimentin and decreased epithelial marker ZO-1 level, while overexpressed CTD903 confirmed these associations. In conclusion, this study shows that LncRNA-CTD903 acts as a tumor suppressor in CRC and can inhibit cell invasion and migration through repressing Wnt/β-catenin signaling, which plays important roles in EMT and CRC metastasis.

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Abbreviations: lncRNA, long non-coding RNA; CRC, colorectal cancer; mCRC, metastatic colorectal cancer; EMT, epithelial-mesenchymal transition; RFS, recurrence-free survival; ZO-1, zonula occludens-1; EGFR, epidermal growth factor receptor; CTD903, CTD-2314B22.3-006, 903 bp; RTCA, real-time cellular analysis

Key words: long non-coding RNA, CTD903, cell migration, epithelial-mesenchymal transition, Wnt/β-catenin, colorectal cancer

Introduction

Colorectal cancer (CRC) is the third most common cancer and accounts for ~9% of all cancer death, ranking the second cause of cancer death worldwide (1). Over 50% of patients with CRC will develop metastasis and the median survival time of metastatic CRC (mCRC) is only 24 months, although various modalities of treatments including chemotherapy with anti-EGFR (epidermal growth factor receptor) targeted monoclonal antibodies have been applied (2-4). Therefore, early diagnosis and prediction of potential metastasis or recurrence show the greatest potential for mCRC therapy. There are a few of tumor biomarkers currently applied for prediction of CRC prognosis, such as gene mutations status of Kras, Braf, PI3K, TP53, and microsatellite instability (MSI) for selection of PD-1 inhibitor in the literature (4-6). It is still pivotal to find additional molecular biomarkers for clinical translation in CRC, especially those that can predict potential tumor recurrence or metastasis.

Except for the only 2% protein-coding genes, the majority in human genome are non-coding genes. Non-coding RNAs were previously regarded as 'noise' or 'junk' RNAs lacking protein-coding potential (7). However, in recent years due to the development of high-throughput sequencing, accumulating evidence revealed that these neglected non-coding RNAs are constitutively expressed and play critical roles in cellular func-

tions and human diseases, such as multigenetic diseases and tumors (8,9). Although small non-coding RNAs [<200 nucleotides (nt)], such as microRNAs, have been the focus of research in RNA biology, in the past few years, long non-coding RNAs (lncRNAs) were also shown to play important roles in human disease and received most attention (9-11). LncRNAs, defined as cellular RNAs >200 nt in length, lack an open reading frame of an important region (<100 amino acids) and thus code no proteins (8). Dozens of lncRNAs are reported to be potential biomarkers for cancer diagnosis and prognosis prediction in many solid tumors (8,9). Recently, LncRNA-H19, UCA1 and HOTAIR were also reported strongly correlated with CRC metastasis and prognosis (12-14). Therefore, lncRNAs are believed to represent a new category of cancer biomarkers and potential tumor therapeutic targets.

In this study, we report on the lncRNA-CTD903 (Ensemble version: ENST00000553153), a transcript of lncRNA-CTD-2314B22.3 (also known as linc01296 in the gene set) (15). It is located in chromosome 14q11.2. We have abbreviated the name of this transcript as lncRNA-CTD903 based on the length of 903 bp.

We previously identified aberrant expression of lncRNAs between CRC and matched paratumor normal tissues by a microarray analysis (13). Briefly, we selected six pairs of CRC tissues and corresponding paratumor normal tissues and assayed the expression of lncRNAs (30,586 items) and protein-coding mRNAs (26,109 items). Then we found lncRNA-CTD903 expression was markedly upregulated (fold change, 15.73, P<0.001) in CRC tissue. The fold change of lncRNA-CTD903 expression ranked in the second position in thousands of abnormally expressed lncRNAs, only secondary to known H19 according to the microarray data. Thus, we selected it for further research. In this study, we confirm CTD903 is upregulated in CRC tissues, compared to peritumor normal tissues. Importantly, CTD903 serves as an independent prediction factor of favorable prognosis with a longer recurrence-free survival (RFS). Furthermore, cell assays find overexpressed CTD903 could suppress cell invasion and migration by inhibiting epithelial-mesenchymal transition (EMT). Finally, our results indicate that CTD903 might repress Wnt/β-catenin signaling and subsequently inhibit expression of the transcription factors Twist and Snail to affect the EMT process. These findings reveal that CTD903 may be a new biomarker for prognosis and a potential target for treating mCRC.

Materials and methods

Patients and sample collection. The study was approved by Human Medical Ethics Committee of Sun Yat-Sen University (SYSU). Fresh CRC tissues (n=115) and paired paratumor normal mucosa samples (2 cm away from the tumor border), and distant normal mucosa samples (≥5 cm away from the tumor border) were collected from patients after surgery. Informed consent was obtained from patients enrolled in this study. Clinical tissue samples were all confirmed histopathologically and stored in RNAlater solution (Invitrogen, USA) at -80˚C until extraction of total RNAs. Clinicopathological parameters were collected from an online CRC database of this hospital, and we confirmed the information by checking original medical records manually. TNM stage was defined according to the 6th version of American Joint Committee on Cancer (AJCC) staging Manual. Follow-up was conducted according to the guideline of National Comprehensive Cancer Network (NCCN). The recurrence-free survival (RFS) was defined as the time interval from radical surgery to relapse, metastasis or death of cancer.

Cell culture. Human CRC cell lines (RKO, SW480, SW620, Caco2, HCT116, DLD1, and HT29) were all purchased in March 2013 from Chinese Academy of Science, Shanghai, China. All cell lines were routinely cultured in the DMEM or RPMI-1640 medium (Gibco, USA), which were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). All cell cultures were maintained in an incubator at 37˚C with a 5% CO_2 humidity.

Quantitative real-time PCR analysis. Tissues were cut up into small pieces, and then ground by a Tissue Lyser (Qiagen). Total RNAs were extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was conducted with Revertra Ace aPCR RT master mix with gDNA remover (Toyobo, Japan). The quantitative real-time polymerase chain reaction (qPCR) was conducted by using SYBR Green mix (Applied Biosystems, USA) according to the instructions. The conditions were as follows: 95˚C for 10 min; and 40 cycles (denaturation at 95˚C for 15 sec, annealing/extension temperature at 60˚C for 1 min). All experiments were performed in triplicates, including a negative control without template. A melting curve was conducted to analyze the appropriate amplification. Agarose gel (1%) was applied to validate the size of PCR products. The oligo primers were used for lack of a poly(A) tail of CTD903. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was applied as the endogenous reference gene. Relative gene expression was normalized to GAPDH using the $2^{-\Delta\Delta CT}$ method. The primers were as follows: CTD903 forward, 5'-TGGCAGTTTAAGAGTCTGGCA-3'; reverse, 5'-GAAGACTCGGGGATCAAGGT-3'. GAPDH forward, 5'-GACAGTCAGCCGCATCTTCTT-3'; reverse: 5'-AATCCGTTGACTCCGACCTTC-3'. All qPCR assays were performed by an ABI 7500 system (Applied Biosystems).

siRNA transfection. According to the manufacturer's instructions, the optimum cell plating density was explored. Cells were plated in the growth medium supplemented with 10% FBS without antibiotics in a 6-well plate, and then cultured for 24 h until 50-70% of confluent. An appropriate density of $3x10^5$ cells per well were plated, then the volumes of siRNA were used for different cell lines from 2 to 10 μl, to reach ≥70% of decreased expression level after silencing. The optimum volumes of transfected siRNAs for RKO, SW480, and SW620 were 2, 3 and 5 μl, respectively. Moreover, an equivalent volume of scramble control was used. After establishing the optimum volume, siRNA or scramble control was mixed with Lipofectamine RNAiMAX reagent (Invitrogen) in reduced serum medium Opti-MEM (Gibco), and then co-transfected with cells. Forty-eight hours after the transfection, cells were harvested for further studies. The siRNA oligonucleotides were designed and synthesized by Ribobio (Guangzhou, China). The siRNA

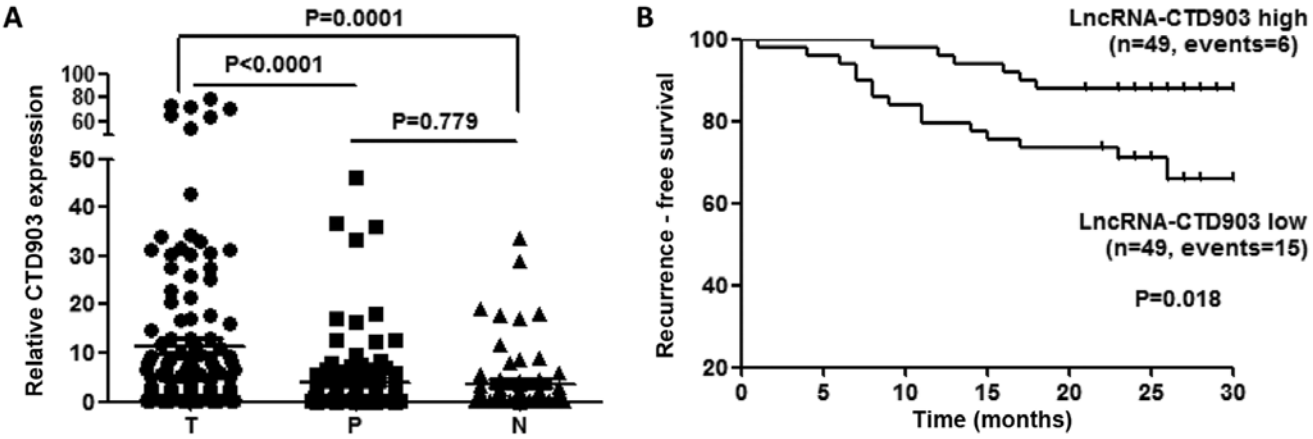

Figure 1. The upregulation of CTD903 level was associated with favorable prognosis of CRC patients. (A) Analysis of CTD903 level was performed in 115 paired CRC (T) and paratumor tissues (P), along with 66 cases of normal intestine tissues (N). The CTD903 levels were normalized to GAPDH in each sample. The results are presented as log10 ($2^{-\Delta\Delta Ct}$). CTD903 expression in T were significantly higher than P (P<0.0001) and N (P=0.0001). No significant difference was found between P and N (P=0.779). (B) Kaplan-Meier analysis of the correlations between CTD903 expressions and recurrence-free survival (RFS) are shown. CTD903 high expression obtained a longer RFS than CTD903 low expression (n=98, log-rank: P=0.018). The median value of CTD903 level was used as the cutoff of high (> median value) and low (≤ median value) expression.

sequences of lncRNA-CTD903 in this study were as follows: forward, 5'-GCUACCUUGUGGCAGUUUAdTdT-3'; reverse, 5'-UAAACUGCCACAAGGUAGCTdTd-3'.

Overexpression of lncRNA-CTD903. To obtain cell lines over-expressing lncRNA-CTD903, the whole length of CTD903 was synthesized by Invitrogen Branch Corp. (Guangzhou, China), and then was inserted into pcDNA3.1(+) plasmid at the multiple cloning site using *Hin*dIII and *Eco*RI restriction enzymes (New England Biolabs) in our laboratory, and then confirmed by sequencing. HCT116 and DLD1, at an appropriate density of 3^x10^5 cells per well, were transfected with 1 μg of plasmid pcDNA3.1-lncRNA-CTD903 (named pcDNA-CTD903 for short) or control pcDNA3.1(+) (named pcDNA for short) using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. The CTD903 levels in overexpressed cell lines were identified by qRT-PCR.

Cell invasion and migration assays. Cell invasion ability was assessed using the cell culture insert (Corning, USA) according to the manufacturer's protocols. An appropriate density of 40-60 thousand cells per well for different cell lines were plated onto membrane (8.0-μm pore size) coated with Matrigel and fibronectin (BD, USA) in the upper chamber of the 24-well insert that contains serum-free medium. The bottom chamber contained growth medium with 20% FBS. Then cells were incubated at 37˚C and 5% CO_2 humidity for 48 h, and then the bottom of the upper chamber insert was fixed with 4% paraformaldehyde and stained with crystal violet. The cells that remained on the inner membrane of the upper chamber were removed by a cotton swab. The images of invaded cells were captured on a 100X inverted microscope (five random fields for each well), and then invaded cells were counted. Cell migration assay was performed with the same method, but without Matrigel. The experiments were repeated at least three times independently.

Cell proliferation, apoptosis and cell cycle assays. Cell proliferation was assessed by cell counting kit-8 (CCK-8) (Dojindo, Japan) and real-time cellular analysis (RTCA) DP device (ACEA Biosciences, USA) at the same time. Briefly, cells were plated in 96-well plate at an appropriate density of 6,000-8,000 cells per well for different cell lines, 10 μl of CCK-8 was added to each well at the indicated time-points and incubated for 2 h. Then the optical density was measured by a multimode spectrum system (Thermo Scientific, USA) at 450 nm. The methods of RTCA cell proliferation assays, apoptosis and cell cycle assays by flow cytometric analysis were described in our previous study (13).

Cell adhesion assays. Cells were plated onto a 96-well plate in triplicate wells coated with fibronectin (BD) at an appropriate density of 10-20 thousand cells per well for different cell lines, and incubated for 30 min with normal growth medium containing 10% FBS. Then cells were washed with PBS two times to remove cells that did not adhere. Adhering cells were fixed with 4% paraformaldehyde, and the images of cells were captured under an inverted microscope (five random x100 fields), and the number of cells was calculated by ImageJ software.

Western blot analysis. Cells were harvested and lysed by RIPA buffer (Beyotime, China) with protease inhibitor (Thermo Scientific). Lysates were then clarified by centrifugation and the concentrations of extracted proteins were measured using BCA Protein Assay kit (Beibo, China). Proteins were incubated with SDS-PAGE loading buffer at 95˚C for denaturation. Proteins (35 μg) were separated by using 5% stacking gel and 10% running gel. The prestained protein ladder (Thermo Scientific) was loaded in parallel as a reference of protein molecular weight. Then proteins were transferred onto NC membranes (Millipore, USA). The blots were blocked with 5% non-fat milk (BD) in TBS with Tween-20 (TBST). Then the membranes were incubated with primary antibody at 4˚C overnight. After washing with TBST, secondary antibodies

HRP-labeled IgG (Abcam, USA) were incubated for 1 h, and then the bound antibodies were detected using enhanced chemiluminescence system (Odyssey, USA). Quantitative analysis was performed by ImageJ software. Protein levels were all normalized to β-actin. The primary antibodies used in this study were as follows: E-cadherin, N-cadherin, Vimentin, ZEB1, Snail, and ZO-1 from an EMT kit (Cell Signaling Technology, USA), β-catenin (BD), Twist 2 (Abcam), β-actin (Proteintech Group, USA).

Statistical analysis. All of statistical analyses were performed by SPSS version 17.0 software (Chicago, IL, USA). For the comparisons, Student's t-tests or Chi-square test was performed appropriately. Potential risk factors of RFS were evaluated by univariate analysis. These risk factors with P<0.10 in univariate analysis were included in the subsequent multivariate analysis using Cox proportional hazards model. All P-values reported were two-sided and the difference with a P<0.05 was considered to be statistically significant.

Results

The novel lncRNA-CTD903 expression is upregulated in CRC tissues. We analyzed CTD903 expression in 115 pairs of CRC tissues (T) and corresponding paratumor tissues (P) using qRT-PCR analysis. The distant normal intestine mucosas (N) from 66 cases of these 115 patients were also assessed. We found that the CTD903 expression was upregulated more obviously in T than that in P (P<0.0001) and N (P=0.0001), while no significant difference was found between P and N (P=0.779) (Fig. 1A).

The correlations of CTD903 and clinicopathological parameters and prognosis. We further examined the correlation of CTD903 expression and clinicopathological data in 115 CRC patients. Patients were categorized as high or low CTD903 expression group according to the median value. The higher CTD903 group showed more percentages of colon cancers comparing with rectum cancers (P=0.031), less mucinous tumors (P=0.030), and smaller tumor size (P=0.041). No significant correlations between CTD903 expression and other clinicopathological parameters were observed, such as age, gender, differentiation, stages, lymphatic and distant metastasis, venous and nerve invasion (Table I).

To explore whether CTD903 expression could influence the clinical outcomes of CRC patients, we plotted ~2-year RFS curves in two independent cohorts. In the total of 115 patients, 17 were excluded for RFS analysis, which included five lost to follow-up, six died of non-cancerous causes, and six who had pre-operative distant metastasis and did not undergo radical resection of metastatic sites. Ninety-eight patients were enrolled in final survival analysis, the results showed CTD903 high expression group had a significantly longer RFS (P=0.018) than CTD903 low expression (Fig. 1B), using the median value of CTD903 expression as the cutoff similarly to previous studies (11,16). Univariate analysis showed that CTD903 expression (P=0.066), lymphatic metastasis (P<0.001), distant metastasis (P<0.001), mucinous invasion (P=0.013), venous invasion (P<0.001) and AJCC stages (P<0.001) were prognostic indicators of CRC (Table II). Furthermore, Cox's multivariate

Table I. The correlations between lncRNA-CTD903 and clinicopathological characteristics in 115 CRC.

	LncRNA-CTD903 expression		
Characteristics	High (n=55)	Low (n=60)	P-value[a]
Age (years)			0.424
<60	27	25	
≥60	28	35	
Gender			0.945
Male	37	40	
Female	18	20	
Location			0.031
Rectum	11	23	
Colon	44	37	
Colon cancer			0.708
Left-sided	31	19	
Non left-sided	13	18	
Histologic grade			0.692[b]
Well/moderately	48	49	
Poorly/others	4	2	
Mucinous cancer			0.030
Yes	4	13	
No	51	47	
Tumor size			0.041
≥5 cm	18	30	
<5 cm	37	28	
Depth of invasion			0.898
T1/T2	6	7	
T3/T4	49	53	
Lymphatic node stage			0.708
N0	35	33	
N1	11	14	
N2	9	12	
Distant metastasis			0.503
Present	8	11	
Absent	47	46	
AJCC stage			0.379
I/II	32	30	
III/IV	23	30	
Venous invasion			0.323
Present	8	13	
Absent	47	47	
Nerve invasion			0.923
Present	7	8	
Absent	48	52	

[a]Chi-square analysis; [b]Chi-square analysis with continuity correction.

Table II. Univariate analysis of clinicopathological parameters for recurrence-free survival.

Variable	Hazard ratio	95% CI	P-value
Age (≥60/<60 years)	1.153	0.518-2.568	0.727
Gender (male/female)	0.930	0.411-2.106	0.863
Lymphatic metastasis (positive/negative)	7.058	2.928-17.014	<0.001
Differentiation (well+moderate)/(poor+other)	0.047	0.000-1666.333	0.561
Distant metastasis (yes/no)	4.959	2.126-11.564	<0.001
T stages (T3+T4)/(T1+T2)	3.743	0.506-27.678	0.196
Mucinous invasion (yes/no)	3.052	1.270-7.335	0.013
Venous invasion (yes/no)	6.3111	2.742-14.524	<0.001
Nerve invasion (yes/no)	2.370	0.813-6.914	0.114
AJCC stage (III+IV)/(I+II)	7.354	2.916-18.545	<0.001
Tumor size (≥5 cm/<5 cm)	1.371	0.926-2.030	0.115
CTD903 expression (low/high)	2.156	0.950-4.892	0.066

CI, confidence interval; N, number of patients; AJCC, American Joint Committee on Cancer.

Table III. Multivariate analysis clinicopathological parameters for recurrence-free survival.

Variable	Hazard ratio	95% CI	P-value
Lymphatic metastasis	1.079	0.983-1.185	0.108
Distant metastasis	3.808	1.297-11.177	0.015[a]
Mucinous cancer	2.251	0.796-6.368	0.126
Venous invasion	2.052	0.718-5.863	0.179
AJCC stage (III+IV)	2.670	0.811-8.794	0.106
CTD903 low expression	3.430	1.283-9.167	0.014[a]

CI, confidence interval; AJCC, American Joint Committee on Cancer; [a]P<0.05.

proportional hazards model revealed that CTD903 low expression [HR: 3.430 (1.283-9.167), P=0.014] and distant metastasis [HR: 3.808 (1.297-11.177), P=0.015] were two independent prognostic factors associated with tumor recurrence (Table III). Taken together, these above data suggested the important role of CTD903 in CRC carcinogenesis and metastasis.

Reduced level of LncRNA CTD903 enhances cell invasion/migration and alters cell morphology. To evaluate the effects of CTD903 on cellular behavior, we first examined CTD903 expression in a variety of CRC cell lines, and then selected cell lines for subsequent experiments. For silencing the expression of CTD903, cell lines with relatively higher CTD903 expression including RKO and SW480 were chosen, along with SW620 derived from metastasis. For the silencing assays, cells were treated with CTD903 siRNA or scramble siRNA controls. CTD903 levels were significantly reduced in RKO, SW480 and SW620 after siRNA treatments, compared to controls (Fig. 2A, D and G). Transwell assays showed that both cell invasion and migration were remarkably increased in RKO, SW480 and SW620 after silencing CTD903, compared to controls (Fig. 2B, C, E, F, H and I). Furthermore, after siRNA transfection, cells exhibited more elongated and spindle-like appearance in RKO and SW480, the typical mesenchymal cell morphology, which indicated that CTD903 silencing could induce EMT-like phenotypes (Fig. 3A and B). Reduced adhering cells were also observed by cell adhesion assays after knockdown of CTD903 expression in SW480 (Fig. 3C and D) and SW620 (Fig. 3E and F). Cell proliferation, apoptosis rate and cell cycle profile were also analyzed, but no significant changes of these cell biological functions were observed after silencing CTD903 expression (data not shown). These results suggest reduced level of CTD903 would not perturb the cell division *per se*.

Overexpression of CTD903 impairs cell invasion, migration and proliferation. To elucidate the effects of CTD903 overexpression on cell biological functions, HCT116 and DLD1 with relatively low CTD903 expression were transfected with pcDNA3.1-CTD903 or control pcDNA3.1 plasmids. CTD903 levels were greatly elevated after overexpression of CTD903 both in HCT116 and DLD1 (Fig. 4A and D). Transwell assays revealed that cell invasion and migration were both impaired after transfection in DLD1 (Fig. 4B and C) and HCT116 (Fig. 4E and F). Cell adhesion was increased in HCT116 (Fig. 5A and B). Obvious decreased cell proliferation rate was observed both in DLD1 (Fig. 5C and D) and HCT116 (Fig. 5E and F) after CTD903 overexpression. Taken together, these results showed CTD903 has the ability to inhibit cell invasion and migration and therefore it is reasoned to be a tumor

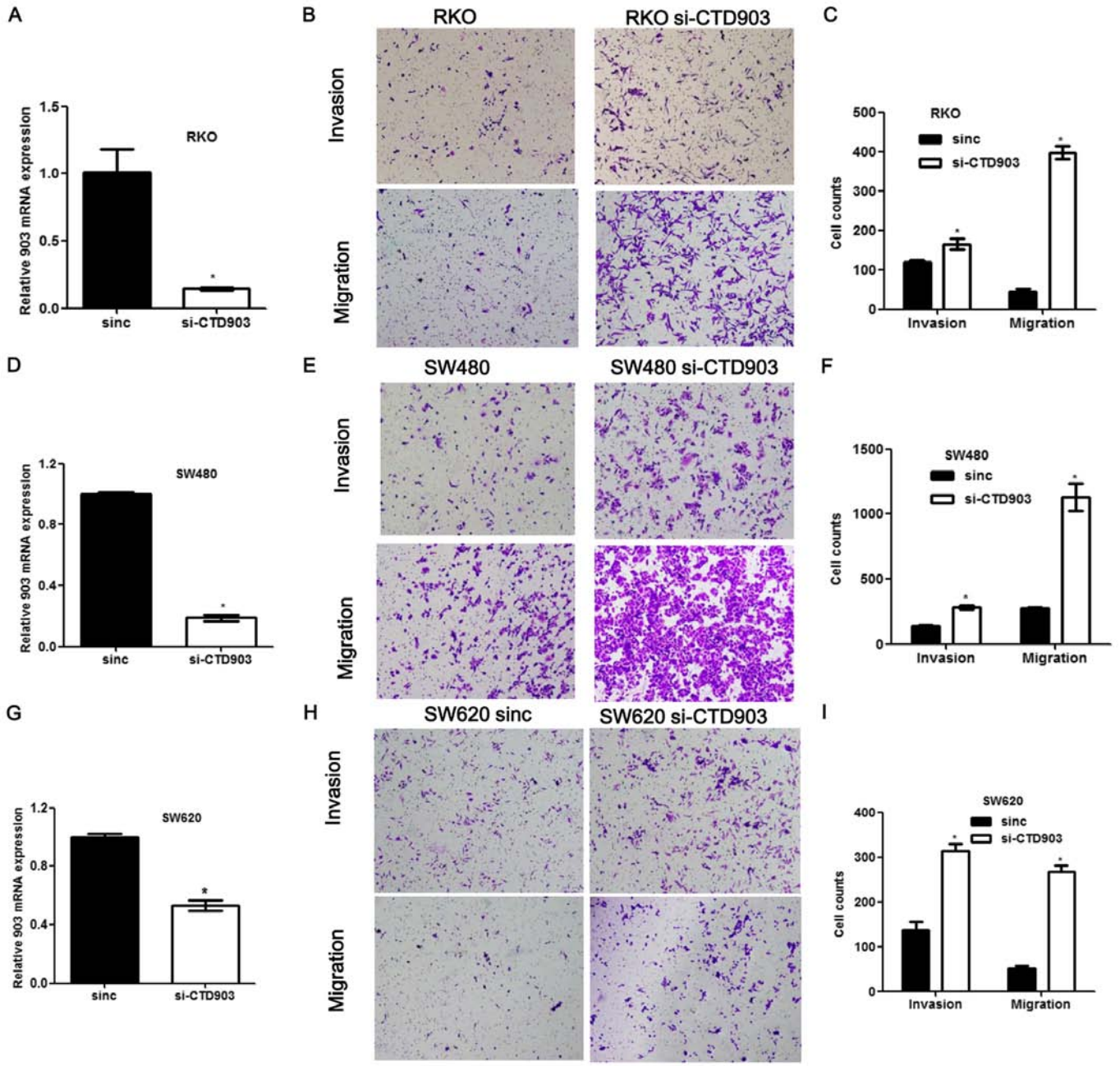

Figure 2. Inhibition of CTD903 expression enhances cell invasion and migration. CTD903 expression was silenced efficiently after transfection of CTD903 siRNA (si-CTD903) or control siRNA (sinc) in RKO (A), SW480 (D), and SW620 (G). Both cell invasion and migration were increased after silencing CTD903 in RKO (B and C), SW480 (E and F), and SW620 (H and I). The number of invaded cells is shown. Data are shown as the mean ± SD, and based on three independent experiments. *P<0.05.

suppressor gene, according to its silencing and overexpression as indicated above.

CTD903 represses Wnt/β-catenin signaling and inhibits EMT-associated transcription factors Twist and Snail expression. To explore the effects of CTD903 on EMT-associated proteins, the expression of epithelial markers were detected by western blotting. After silencing CTD903 in SW480 and SW620, the levels of β-catenin, vimentin, and snail increased significantly, while ZO-1 protein level decreased (Fig. 6A and B). However, no significant changes of E-cadherin (Fig. 6A and B), N-cadherin and ZEB1 protein levels were observed (data not shown). Overexpression of CTD903 in DLD1 and HCT116 cells, decreased levels of β-catenin, Twist and Snail, along with slightly increased ZO-1 expression (Fig. 6C and D), and still no obvious changes of E-cadherin (Fig. 6C and D), N-cadherin and ZEB1 expression (data not shown) were detected. As for the inconsistent changes between E-cadherin and β-catenin expression, previous studies have explained that the reason is E-cadherin was not essential for activating Wnt/β-catenin signaling to play roles in EMT (17). We speculated that knockdown of CTD903 can activate Wnt/β-catenin signaling independently of cadherin expression.

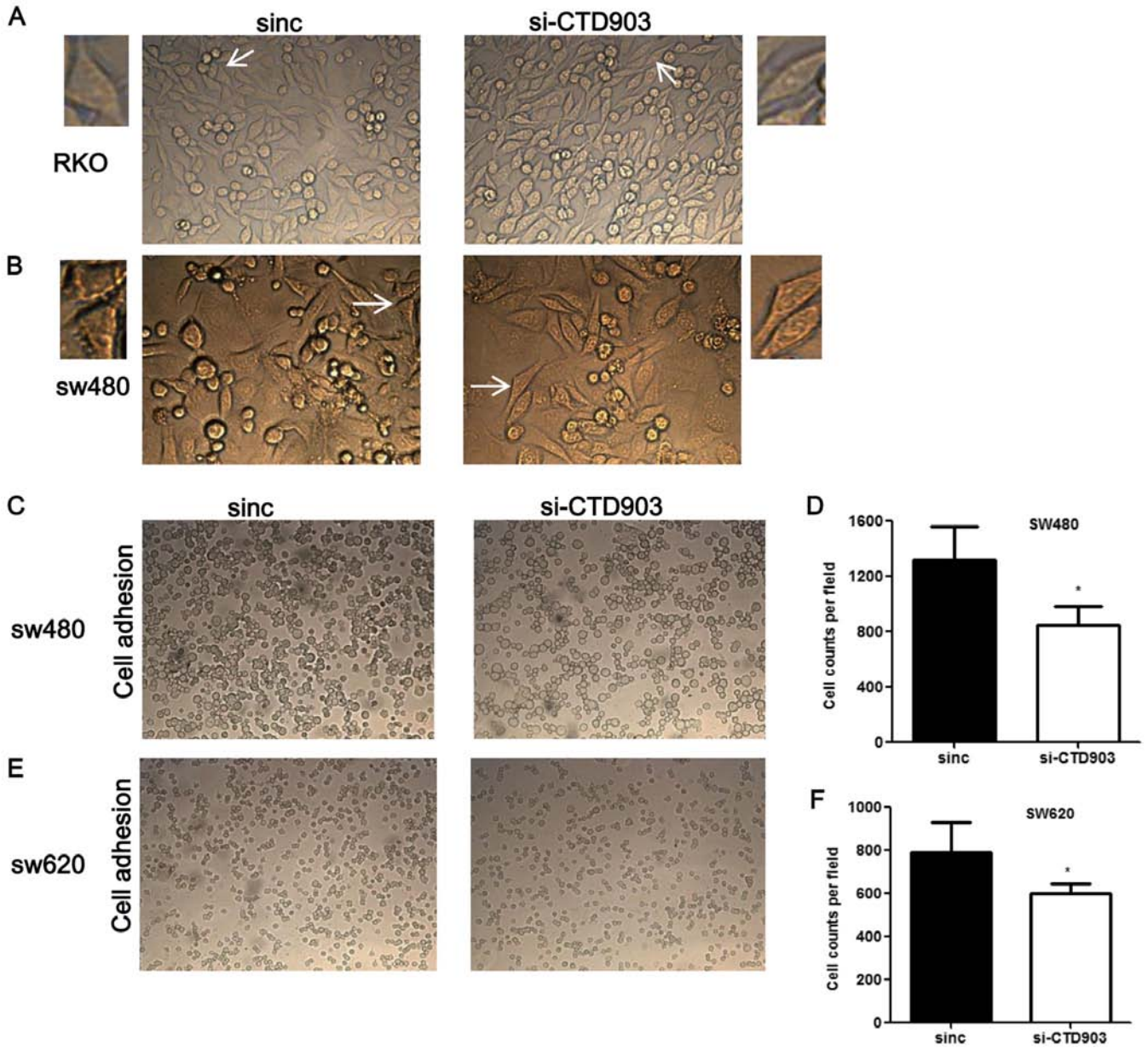

Figure 3. Cell morphology and adhesion were affected by silencing of CTD903. (A and B) Forty-eight hours after transfection of CTD903 siRNA, cells exhibited more elongated and spindle-like appearance in RKO (A) and SW480 (B), compared with the control. Cell morphology was captured at x400 magnification under a light microscope. The insert besides each panel is the typical cell morphology that was picked from the panel (arrows). (C and D) Downregulation of CTD903 levels decreased cell adhesion in SW480 (C and D) and SW620 (E and F). The number of adhering cells is shown. Bars represented adhering cells and are shown as the mean ± SD (n=3). *P<0.05.

Discussion

The human transcriptome is proved to be more complex than known protein-coding genes, while ~90% of the genome is non-coding transcripts (18). Newly discovered lncRNAs have shown important roles in diverse biological functions and tumors in recent years. The underling mechanisms are mainly focused on regulating the processes of gene expression, transcription and translation (8). LncRNA-H19 can increase cell invasion and metastasis in bladder cancer through associating with EZH2 and inhibiting E-cadherin (19), while LncRNA-EBIC is found to promote tumor invasion by similar mechanism in cervical cancer (18). Aberrant expressed lncRNAs also unravelled their importance in CRC (20-25). However, most of lncRNA studies focused on correlations of clinicopathological characteristics and prognosis, the mechanisms are rarely clarified, especially for mCRC.

To the best of our knowledge, we are the first to report the function and mechanism of CTD903 in CRC. In this study, CTD903 overexpression is found and is associated with smaller tumor size, less mucinous adenocarcinomas, and favorable prognosis in CRC, although prognosis did not reach statistical significance due to the small sample size. These results reveal that tumors with CTD903 high expression have less aggressive biological behavior than low expression tumors. CTD903 acts as a tumor suppressor gene and play protective roles in CRC.

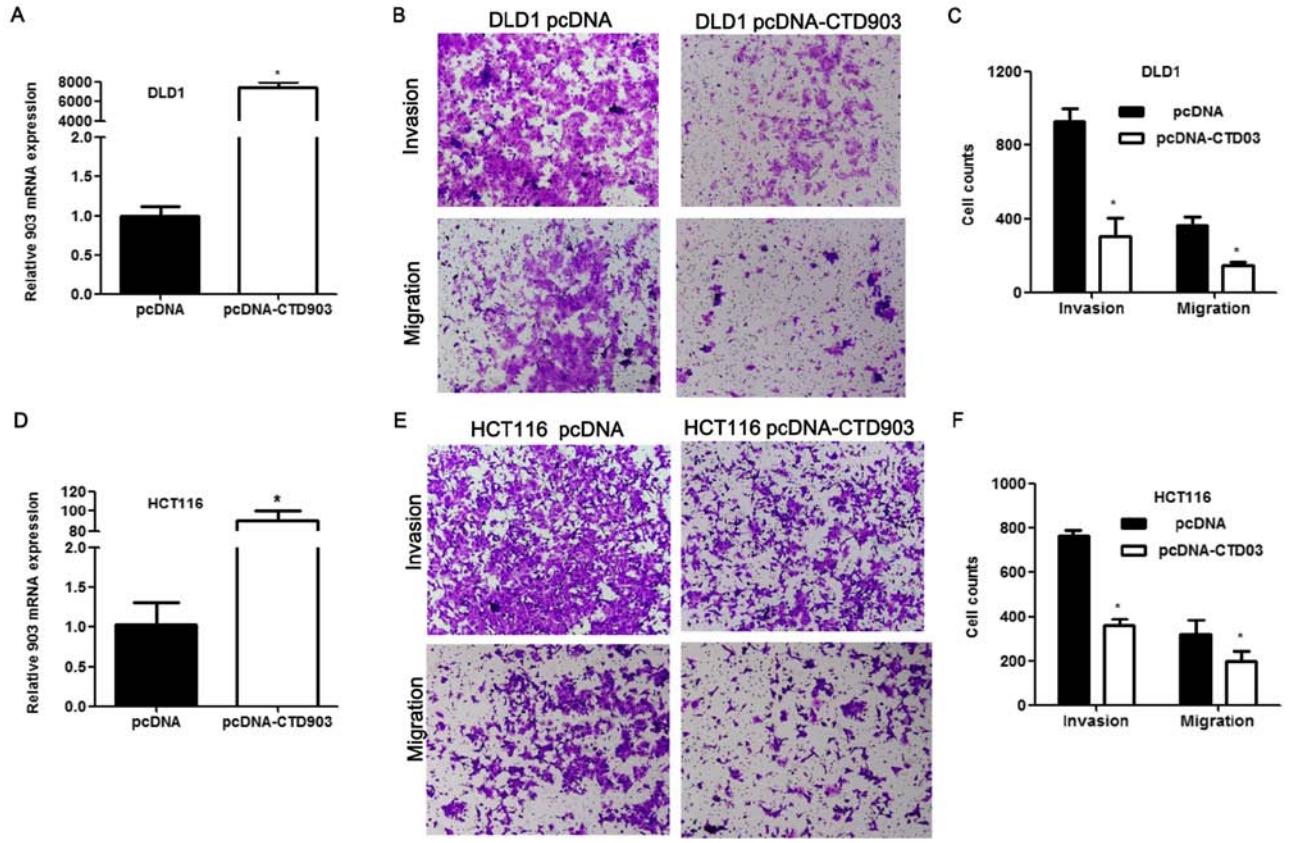

Figure 4. Overexpression of CTD903 expression decreases cell invasion and migration. The pcDNA-903 or pcDNA control was transfected. CTD903 levels were increased significantly after transfection in DLD1 (A) and HCT116 (D) cells. Overexpression of CTD903 decreased cell invasion and migration by Transwell assay in DLD1 (B and C) and HCT116 cells (E and F). The number of invaded cells is shown. Data are shown as mean ± SD. Profiles represent at least three independent experiments. *P<0.05.

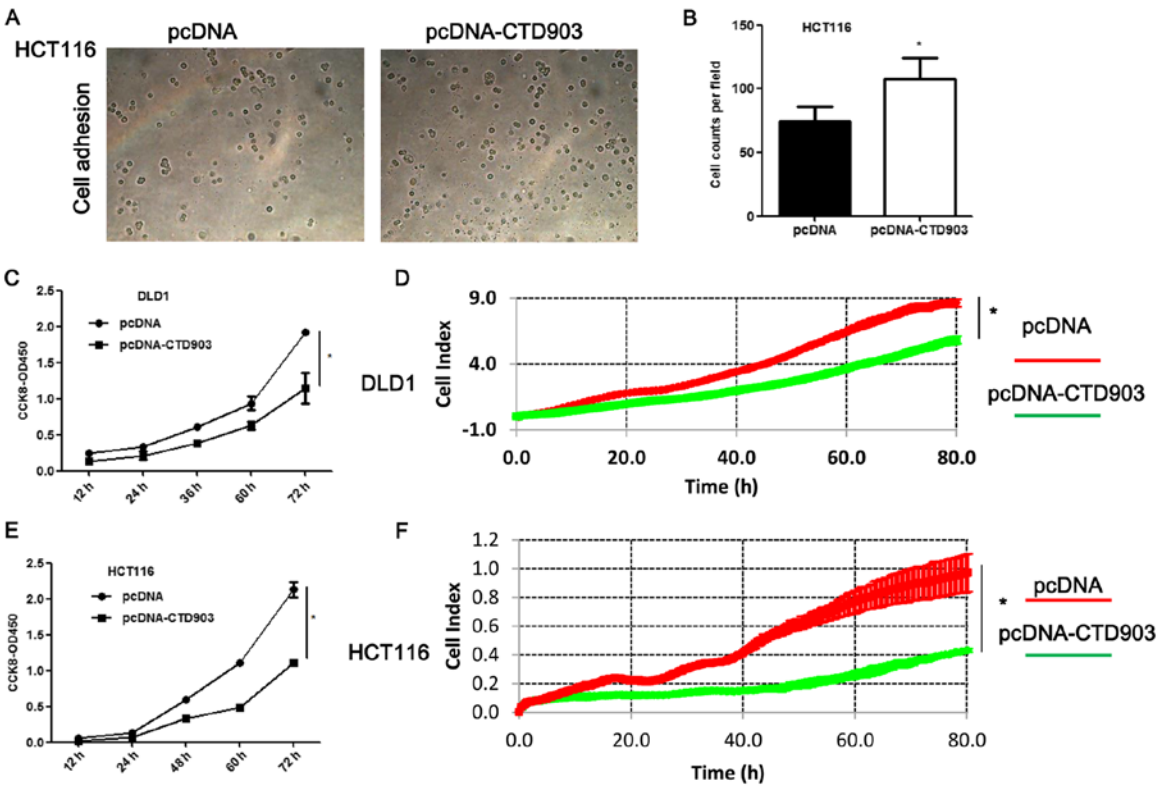

Figure 5. Overexpression of CTD903 increases cell adhesion and decreases cell proliferation. After upregulation of CTD903, cell adhesion was increased in HCT116 (A and B), and cell proliferation rates were inhibited as detected by CCK-8 and RTCA real-time system in DLD1 [(C) CCK-8; (D) RTCA] and HCT116 [(E) CCK-8; (F) RTCA]. Bar, mean ± SD (n=3). *P<0.05.

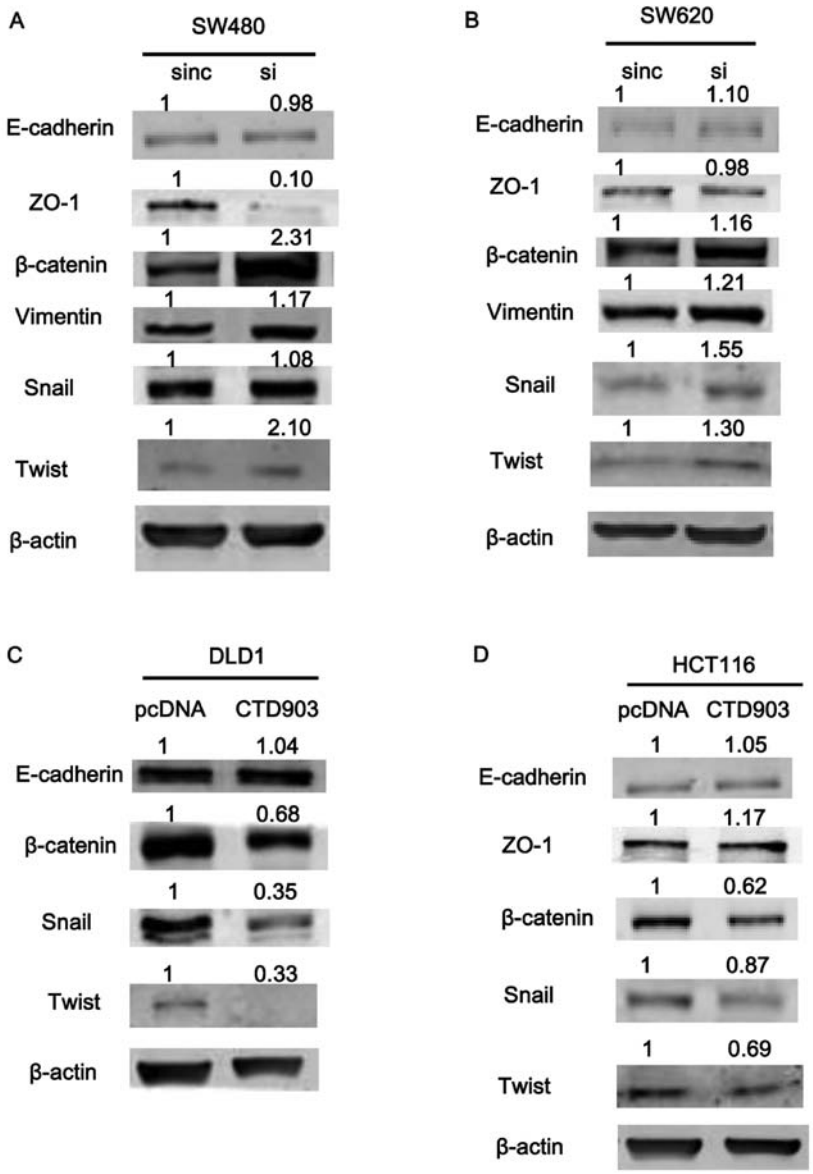

Figure 6. CTD903 inhibits Wnt/b-catenin signaling and affected expression of Twist, Snail, Vimentin and ZO-1. Protein levels of EMT related markers were detected. Inhibition of CTD903 (si) increased levels of β-catenin in Wnt signaling, mesenchymal marker Vimentin, transcription factors (Snail and Twist), and decreased epithelial marker ZO-1 in SW480 (A) or SW620 (B), compared to control (sinc). Overexpression of CTD903 decreased the levels of β-catenin, Snail and Twist in DLD1 (C) or HCT116 (D), compared to control (pcDNA). No significant changes of E-cadherin level were detected. Quantitative results of each band are listed, compared to si or pcDNA control. Profiles represented at least three independent experiments for each cell line.

We also found the parameters, including lymphatic and distant metastasis, mucinous and venous invasion, and AJCC stages in univariate analysis, which are known indicators of poor CRC outcome, and in turn reveal the reliable results of our study. Furthermore, CTD903 is found to be an independent prognostic factor for RFS. Thus, we believe CTD903 might be a potential novel biomarker for future clinical translation.

Recently Qiu and Yan also reported that linc01296 was upregulated and can predict favorable prognosis in CRC by conducting a meta-analysis of online databases in European population (15). Their finding was also consistent with our results. However, the function and underlying mechanism of this lncRNA in tumors remain unknown. Furthermore, as many as sixteen transcripts of linc01296 are documented, but only three of them are overexpressed and others were not aberrantly expressed in CRC according to our previous microarray data, which indicate dramatic heterogeneity among these transcripts (13). CTD903 is the most overexpressed transcript of these three, and ranks in the second position in thousands of aberrant expressed lncRNAs in CRC. Therefore, CTD903 has shown biomarker potential and absorbed our interest for further research.

EMT is characterized by the impairment of cell-cell adhesion and can increase cell motility to promote cancer progression and metastasis (26). EMT is induced through activation of Wnt signaling (27). β-catenin is the key initial protein in the Wnt signaling. After activation, β-catenin can translocate from the cytoplasm to the nucleus, then regulate expression of several transcription factors, and subsequently induce EMT (17,28,29). In accordance with previous

studies, in this study, CTD903 overexpression can repress Wnt/β-catenin expression, and then inhibits expressions of downstream mesenchymal marker Vimentin, epithelial marker ZO-1 and the transcriptional factors Twist and Snail. Finally, phenotypes of EMT and cell invasion and migration are inhibited.

E-cadherin and N-cadherin are two key molecules for EMT. Loss of E-cadherin expression and overexpression of N-cadherin are usually observed when EMT occurs (30). However, E-cadherin is not essentially correlated with activation of Wnt/β-catenin, because other compartments such as APC or AXIN can also confer β-catenin into the nucleus to induce EMT (17). In our study, no significant expression changes of E-cadherin and N-cadherin were observed, neither when EMT was induced. Thus, the results provide additional evidence that activation of Wnt/β-catenin signaling can be independent and not triggered by E-cadherin/N-cadherin. Further research is needed to elucidate the underlying mechanism. No change of ZEB1 expression was observed, which indicated that it may not be involved in CTD903-mediated EMT.

Although most of the reported oncogenes are overexpressed in cancer, small proportion of tumor suppressor genes can also be upregulated and predict good prognosis, such as Rab27A in CRC and FOXO3a in gastric cancer (31,32). In this study, the level of CTD903 is upregulated significantly and is also suggested to be a tumor suppressor gene. In addition, overexpressed CTD903 level can decrease cell proliferation in HCT116 and DLD1, but no increased cell proliferation was observed when CTD903 was knocked down. The reason is perhaps for the limited effects of CTD903 on cell proliferation, like others reported in lncRNA-EBIC, and microRNA-10b that can remarkably affect cell migration but have no effects on cell proliferation (18,30). Cell cycle and apoptosis are not affected in knockdown of CTD903, which may be explained by limited roles on cell cycle and apoptosis, similarly to many other lncRNAs (18,33). Thus, we focus on the invasion and migration to elucidate potential mechanisms of tumor metastasis, which is currently a popular issue (34,35) and the leading cause of cancer-related death that remains not well answered. The most important limitations of this study are lack of *in vivo* experiments, which may provide more robust evidence for our findings.

In conclusion, lncRNA-CTD903 acts as a tumor suppressor gene in CRC, and can repress cell invasion and migration by repressing Wnt/β-catenin signaling to inhibit EMT and CRC metastasis. Our study enriches the underling molecular mechanisms of carcinogenesis and metastasis, and provides a novel biomarker and potential therapeutic target for CRC, which is promising for precision medicine.

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