ZNF281 Regulates Cell Proliferation, Migration and Invasion in Colorectal Cancer through Wnt/β-Catenin Signaling

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Key Words
ZNF281 • Colorectal cancer • Proliferation • Migration • β-catenin

Abstract
Background/Aims: Zinc Finger Protein 281 (ZNF281) was recently identified as a novel oncogene in several human carcinomas. However, the clinical significance of ZNF281 in colorectal cancer (CRC) and the molecular mechanisms by which ZNF281 promotes the growth and metastasis of CRC remain unknown. Methods: ZNF281 expression in CRC tissues was assessed, and the outcomes were analyzed to determine the clinical importance of ZNF281 expression. Cell Transwell assays and a wound healing assay were performed to assess the effects of ZNF281 on CRC cell migration and invasion in vitro. Western blotting was applied to analyze the potential mechanisms. Results: ZNF281 mRNA and protein levels were significantly increased in CRC tissues compared with normal colon tissues, and high ZNF281 expression was associated with advanced T stage, N stage, TNM stage and differentiation. Therefore, ZNF281 expression might be an independent prognostic indicator in CRC patients. Moreover, knockdown of ZNF281 expression suppressed cell proliferation, migration and invasion by inhibiting the Wnt/β-catenin pathway. Conclusion: Our study indicates that ZNF281 plays a critical role in the progression and metastasis of CRC and could represent a potential therapeutic target for CRC.

Introduction

Colorectal cancer (CRC) is one of the most common malignancies and is a leading cause of cancer-related death worldwide [1]. Although the morbidity and mortality rates have declined in some Western countries, these rates have remained high in China and other developing countries [2, 3]. Over the past few decades, many patients have died from cancer...
metastasis and recurrence despite comprehensive treatment strategies including surgery, chemotherapy and radiotherapy [4]. Therefore, studies on the molecular mechanisms underlying the invasion and metastasis of CRC might help improve the treatment and prognosis of CRC patients.

Zinc finger protein 281 (ZNF281, also known as ZNP99 or ZBP99) is located at chromosome 1q32.1 and is a critical regulator of embryonic stem cell differentiation and tissue development [5]. Given that ZNF281 binds to GC-rich regions located in the promoters of a variety of genes, it was first named GC-box-binding zinc-finger protein (GZP1) [6]. Several studies have demonstrated the diverse roles of ZNF281 in stem cell pluripotency and development processes [7-9]. Therefore, ZNF281 is regarded as a relevant gene for cellular stemness. Notably, recent investigations have revealed that ZNF281 is a novel oncoprotein that is frequently overexpressed in human malignancies, including breast, renal and pancreatic carcinomas [9-11]. More importantly, high ZNF281 expression in cancer tissues correlates with advanced clinical stage, enhanced metastatic potential and reduced patient survival [9, 10]. More recently, an interesting finding demonstrates that ZNF281 expression is closely associated with more aggressive cancer characteristics and a poor prognosis of patients with CRC [12]; however, its actual role and underlying mechanism in CRC invasion and metastasis have not yet been elucidated.

The Wnt/β-catenin signaling pathway is involved in a variety of cell processes, including cell proliferation, differentiation and embryonic development [13, 14]. As suggested by an increasing number of studies, the abnormal activation of Wnt/β-catenin signaling is associated with various types of cancer cells [15-17]. Clinical statistics indicate that aberrant Wnt/β-catenin signaling is typically accompanied by oncogenic activation, which is commonly observed in CRC patients [18]. Another study clarified that ZNF281 may promote pancreatic cancer cell proliferation and invasion by interacting with the Wnt/β-catenin pathway and that such interactions may be associated with the progression of pancreatic cancer [10]. As a result, we aim to assess whether the abnormal expression of ZNF281 or β-catenin is involved in the progression of CRC, thereby revealing their potential molecular functionality.

In this study, we investigated the potential involvement of ZNF281 in CRC cell proliferation, migration and invasion. We assessed whether Wnt/β-catenin is involved in the biological activities of ZNF281 in the context of CRC. Here, we found that ZNF281 was upregulated in CRC and that its expression levels were positively correlated with increased invasion and migration potential of CRC cells. Our data suggested that ZNF281 may be a potential therapeutic target for CRC.

**Materials and Methods**

**Patients and tissue specimens**

Resected specimens were collected from patients with primary CRC who underwent gastrointestinal surgery between January 2001 and December 2003 at the Huai He Hospital of He Nan University. The diagnosis was confirmed by at least two pathologists, and tumor staging was based on pathological findings according to the American Joint Committee on Cancer (AJCC) guidelines. Freshly obtained cancer tissues and adjacent normal mucosa (10 cm from the original tumor site) were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA and protein extraction. Follow-up continued until December 2017. The Institutional Ethics Review Board of the Huai He Hospital of He Nan University approved the use of all tissue blocks and serum samples for this study, and written consent was obtained from each patient.
Bioinformatics analysis

RNASeq data for CRC were downloaded from The Cancer Genome Atlas (TCGA) database (https://genome-cancer.ucsc.edu) [19, 20]. ZNF281 mRNA expression levels in CRC tissues and adjacent normal colon tissues were obtained and analyzed.

Cell culture and retroviral infection

The human colon cancer cell lines SW620, HT29, HCT116, LoVo, DLD-1 and SW480 were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco’s Modified Eagle Medium, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1% penicillin/streptomycin. All cells were maintained in a humidified atmosphere at 37°C containing 5% CO₂.

ZNF281 shRNA and the corresponding negative control shRNA were purchased from the RiboBio Co., Ltd. (Guangzhou, China). SW480 and HCT116 cell infections with human ZNF281 shRNA were performed as previously described [20]. Nonsilencing (vector) shRNA was used as a negative control. The knockdown efficiency of ZNF281 was examined by quantitative qRT-PCR and Western blot.

Immunohistochemical (IHC) staining

IHC staining was analyzed as previously described [21]. Briefly, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. Heat-mediated antigen retrieval was then performed with sodium citrate buffer (10 mM, pH 6.0). Tissue sections were heated in a microwave for 20 min. Endogenous peroxidase activity was suppressed by exposure to 3% hydrogen peroxide for 10 min. Slides were then blocked in 5% BSA (Boster Bioengineering, Wuhan, CN) and incubated with diluted (1:100) primary ZNF281 antibody (Abcam, Cambridge, MA, USA) for 1 h at 37°C. Slides were then incubated with diluted secondary antibody (1:2000) (Abcam, USA) for 20 min at 37°C. Slides were visualized with DAB and were counterstained with hematoxylin for microscopic examination. For the semiquantitative analysis of staining intensity, a scoring method [22] (H-score) was used with minor modifications. Immunohistochemical evaluation was performed by two pathologists who were blinded to the clinical and pathological characteristics of the specimens.

Western blot

Total protein was extracted with cell lysis buffer (KeyGene, Nanjing, China), and the protein concentration was quantified using an Enhanced BCA Protein Assay Kit (KeyGene, Nanjing, China). The proteins were separated by 8-10% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, Billerica, MA, USA). The membrane was blocked for 1 h in 5% BSA in TBS-T and probed with the corresponding primary antibodies overnight at 4°C, which was followed by incubation with rabbit and mouse horseradish peroxidase-coupled secondary antibodies for 1 h. Specific bands were detected on autoradiographic film using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using RNA Plus Reagent (TaKaRa, Japan). Complementary DNA was prepared using oligo T primers according to the protocol supplied with the PrimeScript™ RT Reagent Kit (TaKaRa, Japan). ZNF281 expression was determined by quantitative real-time PCR using Power SYBR Green PCR master mix (Applied Biosystems, USA).

Proliferation assay

The Cell Counting Kit-8 (CCK-8) assay was used to detect cell proliferation. Briefly, cells were seeded onto 96-well cell culture plates (KeyGene, Nanjing, China) at a density of 3×10⁵ cells/well in 100 μL of culture medium. Then, 10 μL of CCK-8 reagent (Dongjido, Japan) was added to each well for a 2 h incubation at 37°C according to the manufacturer’s instructions. The absorbance was read at a wavelength of 450 nm with an automated plate reader (M200 Pro, Tecan). The experiments were repeated at least three times.
Flow cytometry analyses of the cell cycle

Cell cycle analyses by flow cytometry were performed as previously described [21]. Briefly, cells were seeded in six-well plates and cultured for 48 h. Next, cells were harvested, washed with PBS, and treated with cold 70% ethyl alcohol at 4°C overnight. The cells were then washed with PBS again and treated according to the instructions of the cell cycle analysis kit (Beyotime, China). Cell cycle analysis was performed on the Accuri C6 system (BD Biosciences, Mountain View, CA, USA).

Wound healing assay

Infected SW480 and HCT116 cells were seeded into six-well plates and cultured until they were completely confluent. The cells were then starved overnight in serum-free RPMI-1640 medium. Artificial wounds were generated using a sterile 200 µL pipette tip, and the cells were washed with serum-free medium to remove floating cells and debris. Images were obtained at 0 and 24 h using a microscope (Olympus Corp., Tokyo, Japan). Migration ability was assessed by measuring changes in the size of the wound areas.

Transwell invasion assays

Cell invasion was measured using 24-well Transwell cell culture inserts (Corning, Cambridge, MA, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). Briefly, 1×10^5 infected SW480 or HCT116 cells in serum-free RPMI-1640 medium were placed in the upper chamber of each insert, and medium containing 10% fetal bovine serum was added to the lower chamber. After a 24-h incubation, the cells remaining on the upper surface of the membranes were removed, and cells that had invaded the lower surface were fixed, stained and counted under an inverted microscope (×200) to calculate their relative numbers. All assays were performed in triplicate.

Statistical analyses

SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for the analyses. The relationships between ZNF281 expression and the features of tumor progression were analyzed using the chi-square and Fisher’s exact tests. Kaplan–Meier survival curves were constructed, and the log-rank test was performed for univariate analyses. The multivariate analysis was performed using Cox’s proportional hazards model. A P-value of 0.05 was considered statistically significant for all analyses.

Results

Increased ZNF281 levels in the tumor tissues of CRC patients

To evaluate the expression of ZNF281 in CRC, we first analyzed ZNF281 expression in TCGA cohort datasets (Fig. 1A & B). ZNF281 mRNA levels in the unpaired CRC tumor (N=383) and normal colon tissues (N=50) from the TCGA cohort demonstrated that ZNF281 mRNA expression was significantly upregulated in CRC (P<0.001, Fig. 1A). The upregulation of ZNF281 in CRC was further validated in paired tumor and adjacent tissues in the TCGA cohort (N=32, P<0.001, Fig. 1B). A similar trend was observed in other CRC cohorts [23-28] from the Oncomine database (Table 1). Furthermore, in 30 pairs of fresh specimens from our medical center, ZNF281 mRNA levels were increased in CRC tissues as determined by qRT-PCR (Fig. 1C). Similarly, ZNF281 expression was significantly upregulated in CRC tissues compared with paired normal colon tissues (N=8, Fig. 1D).

Correlation of ZNF281 expression with the clinical characteristics of CRC patients

We evaluated whether ZNF281 protein was associated with the clinical characteristics of CRC patients. To this end, immunohistochemistry (IHC) was performed and ZNF281 expression was scored in 150 CRC tissues and 33 adjacent tissues. Representative ZNF281 IHC staining images are presented in Fig. 2A & B. Of the 150 CRC patients, 19 patients (12.67%) had negative staining (IHC score: 0), 12 patients (8.00%) had weak staining (IHC score: 1), 36 patients (24.00%) had moderate staining (IHC score: 2), and 83 patients (55.33%) had strong staining (IHC score: 3). Negative and weak staining were defined as low ZNF281 expression,
whereas moderate and strong staining were defined as high ZNF281 expression. Based on IHC staining, all patients were divided into a ZNF281-negative group (N=31) and a ZNF281-positive group (N=119). As illustrated in Table 2, a chi-square test suggested that positive ZNF281 expression was significantly correlated with advanced T stage (P=0.001), N stage (P=0.009) and TNM stage (P=0.000). Moreover, positive ZNF281 expression also predicted the differentiation and tumor grade. However, no statistically significant relationships were noted between positive ZNF281 expression and patient gender (P=0.869), age (P=0.677), tumor location (P=0.644), M stage (P=0.095), or vascular invasion (P=0.301).

**ZNF281 upregulation is associated with poor patient prognosis**

We next evaluated the relationship between ZNF281 expression and the prognoses of the CRC patients at our medical center. Kaplan-Meier curves were used to compare survival between the two groups, and it was found that the OS and RFS in the ZNF281-negative group were significantly better compared with those in the ZNF281-positive group (Fig. 2C, P<0.0001 and Fig. 2D, P=0.0003).
As shown in Table 3, univariate analyses revealed that N stage (HR=0.501, 95% CI: 0.303-0.826, P=0.007), M stage (HR=8.622, 95% CI: 2.479-29.988, P=0.001), TNM stage (HR=2.144, 95% CI: 1.007-4.564, P=0.048), and ZNF281 expression (HR=3.352, 95% CI: 1.740-6.457, P=0.000) were significantly associated with CRC patient prognosis. Furthermore, a multivariate analysis demonstrated that ZNF281-positive expression was an independent prognostic predictor among CRC patients (HR=3.325, 95% CI 1.776-6.226, P=0.000).
ZNF281 promotes CRC cell proliferation in vitro

Given that high ZNF281 expression was significantly correlated with a poor prognosis, we then investigated the biological function of ZNF281 in CRC cells. We detected ZNF281 protein (Fig. 3A) and mRNA (Fig. 3B) levels in 6 CRC lines and found that HCT116 and SW480 exhibited relatively high ZNF281 expression. Therefore, we selected the HCT116 and SW480 cell lines for subsequent analysis of the functional role of ZNF281 in CRC. To further examine
the pathobiological effect of ZNF281 in CRC, we silenced its expression in the high ZNF281-expressing cell lines by transfecting the cells with ZNF281-shRNA. ZNF281 expression was detected by Western blot (Fig. 3C) and qRT-PCR (Fig. 3D).

The effect of ZNF281 knockdown on the proliferative ability of CRC cells was assessed using CCK-8 assays. The proliferation rates of HCT116 and SW480 cells were dramatically reduced following ZNF281 knockdown (Fig. 4A). In addition, flow cytometry-based cell cycle analysis also revealed that ZNF281 silencing increased the percentage of cells in G1 phase arrest \( (P<0.001) \) (Fig. 4B). Taken together, our data indicate that ZNF281 could promote CRC cell proliferation *in vitro*.

**Table 3.** Univariate and multivariate analyses of the influence of clinicopathological factors on the prognoses of patients with CRC

<table>
<thead>
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**Fig. 3.** ZNF281 is highly expressed in CRC cell lines and successful knockdown of ZNF281 in SW480 and HCT116 cells. ZNF281 expression was examined by Western blotting (A) and qRT-PCR (B) in the SW620, SW480, LoVo, HT29, HCT116 and DLD-1 cell lines. (C) Western blotting was used to detect ZNF281 knockdown efficiency in SW480 and HCT116 cells. (D) qRT-PCR was used to detect ZNF281 knockdown efficiency in SW480 and HCT116 cells. Mean ± SEM, t-test, **P<0.01, ***P<0.001.
ZNF281 promotes CRC cell migration and invasion in vitro

We next investigated the potential role of ZNF281 in modulating the ability of CRC cells to migrate and invade. The effect of ZNF281 on cell migratory capacity was first analyzed using wound healing assays. The results revealed an apparent decrease in sh-ZNF281 cell migration compared with control cells (Fig. 5A). Next, we determined the role of ZNF281 in tumor cell invasion. Matrigel Transwell assays revealed that lower ZNF281 expression significantly reduced the rate of invasion compared with control cells (Fig. 5B). Therefore, these results demonstrate that the ZNF281 expression levels are positively correlated with CRC cell migration and invasion.

ZNF281 regulates the Wnt/β-catenin signaling pathway

Aberrant activation of Wnt/β-catenin signaling reportedly contributes to the invasion, metastasis and progression of CRC [29]. To determine the involvement of the Wnt/β-catenin signaling pathway in the tumorigenic effects of ZNF281, the expression of β-catenin and a target gene of Wnt/β-catenin (c-myc) were examined by qRT-PCR and Western blot. ZNF281 knockdown reduced β-catenin and c-Myc expression at the mRNA level (Fig. 6A and B). These data imply that upregulation of ZNF281 could activate the Wnt/β-catenin signaling pathway.

To further analyze whether Wnt/β-catenin signaling is involved in the mechanism of ZNF281, we treated the cells with the Wnt/β-catenin pathway inhibitor XAV939. The expression of β-catenin and c-Myc in HCT116 and SW480 cells was significantly decreased after treatment with XAV939 (Fig. 6C). These data suggest that ZNF281 functions as a positive regulator of Wnt/β-catenin signaling, which consequently promotes CRC cell proliferation, migration and invasion.
Discussion

Metastasis is the major cause of cancer-related deaths [30]; therefore, the prevention and treatment of metastasis are fundamental to improving clinical outcomes. Few studies have demonstrated that zinc finger proteins regulate cancer metastasis [31]. ZNF281, a zinc finger transcription factor, belongs to the C2H2-type zinc finger motif, a subfamily of zinc finger proteins. Recent studies have provided new insights into the function of ZNF281 in epithelial-mesenchymal transition (EMT); it has also been reported that ZNF281 regulates numerous EMT-related effector genes [6, 9]. EMT increases cell migratory and invasive abilities, which are associated with the first step of CRC metastasis [32, 33]. Experimental downregulation of ZNF281 expression in a mesenchymal CRC cell line decreased the formation of lung metastases of CRC cells in a xenograft mouse model, which demonstrates a potential role of ZNF281 in cancer metastasis, but the mechanism is completely unknown. In this study, we demonstrate the connection between ZNF281 and CRC metastasis, and we also suggest a possible mechanism for this effect for the first time.

In this study, we detected high ZNF281 expression in a majority of the CRC tissue samples tested, which correlated with tumor differentiation, T stage, N stage, and TNM stage. This observation is consistent with the recent finding by Qian et al., who reported that ZNF281 expression is aberrantly high in pancreatic cancer and is closely correlated with tumor stage [10]. Lymphatic metastasis is an early step of metastasis [34]. In this study, we determined...
that ZNF281 is positively correlated with CRC lymphatic metastasis in clinical samples, which demonstrates its role in cancer metastasis. Similarly, high ZNF281 expression is frequently detected in primary breast cancer tissues with metastatic potential, which implies a role in tumor progression [12]. Moreover, positive ZNF281 expression was predictive of worse OS and RFS in CRC patients compared with negative ZNF281 expression. These results are consistent with the findings of a previous study [8, 35]. Taken together, these results indicate that ZNF281 expression may play an important role in CRC progression.

Inspired by the close correlation between ZNF281 and tumorigenesis, we hypothesized that ZNF281 may also be involved in tumor malignancy. The proliferation, migration and invasion capacity of cells are the three most important features of malignant cell behavior [36]. We then applied a lentivirus-mediated shRNA targeting assay to knock down ZNF281 in CRC cells. The results demonstrated that CRC cell proliferation capacity was remarkably decreased in vitro following ZNF281 knockdown, which might be due to G0/G1 phase arrest.

To further demonstrate the role of ZNF281 in regulating CRC cell migration and invasion, Transwell and wound-healing assays were performed, which demonstrates that ZNF281 silencing inhibited cell migration and invasion in SW480 and HCT116 cells. Our results are consistent with those of a prior of pancreatic cancer study [10]. These results indicate that ZNF281 plays an oncogenic role in CRC development.
β-catenin is involved in tumor progression through activation of the Wnt/β-catenin signaling pathway and its downstream proteins [37]. Aberrant activation of the Wnt/β-catenin signaling pathway, which results in uncontrolled cell proliferation and impaired migration, is a common event in human cancer development [38, 39]. Moreover, 90% of CRC tumors exhibited aberrant Wnt/β-catenin signaling activity [40]. Here, our data suggest that silencing of ZNF281 not only significantly suppressed β-catenin expression but also attenuated the proliferation, migration and invasion of CRC cells.

The coordinated upregulation and downregulation of multiple genes converge to increase tumor cell migration and growth during CRC progression [41]. The c-myc oncogene is identified as a target gene of the Wnt/β-catenin signaling pathway [42, 43]. Studies have shown that there is a direct interaction between ZNF281 protein and c-myc oncoprotein [12, 38, 44]. Furthermore, ZNF281 may also promote β-catenin/TCF4 activity by mediating the loss of E-cadherin expression, which inhibits β-catenin nuclear localization and activity by recruiting it to the cell membrane [12, 45]. In the present study, ZNF281 knockdown strongly inhibited CRC cell proliferation, migration and invasion, which are related to reduced c-myc and β-catenin protein expression in CRC cells. To further confirm the role of ZNF281 in regulating Wnt/β-catenin pathway activity, XAV939 was used to inhibit the Wnt/β-catenin pathway. Our data show that XAV939 markedly reduced the expression of β-catenin and c-Myc in sh-ZNF281 cells. Therefore, these observations indicate that ZNF281 may promote CRC cell growth and migration through Wnt/β-catenin-dependent mechanisms and suggest that ZNF281 can be further evaluated as a potential therapeutic target for CRC.

Conclusion

Our study confirmed that ZNF281 was elevated in colorectal cancer and that ZNF281 expression was an independent prognostic indicator in CRC patients. The silencing of ZNF281 inhibited the proliferation, migration and invasion of colorectal cancer cells through the canonical Wnt/β-catenin signaling pathway. Therefore, ZNF281 could potentially serve as a new therapeutic target or clinical biomarker for metastatic CRC.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

References


