Circ-KRT6C promotes malignant progression and immune evasion of colorectal cancer through miR-485-3p/PDL1 axis

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Running Title Page
Circ-KRT6C/miR-485-3p/PDL1 axis in colorectal cancer.

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Abbreviations: circular RNA (circRNA), programmed cell death receptor ligand 1 (PDL1), cell count kit 8 (CCK8), untranslational region (UTR), Programmed cell death protein 1 (PD-1).
Abstract

**Background:** Recently, circular RNA (circRNA) was reported to be a significant participant in the development of tumorigenesis, including colorectal cancer. Therefore, we aimed to clarify the precise role of circ-keratin 6C (circ-KRT6C) in colorectal cancer progression. **Methods:** The relative expression levels of circ-KRT6C, microRNA-16-5p (miR-485-3p), and programmed cell death receptor ligand 1 (PDL1) were analyzed by real-time quantitative polymerase chain reaction and western blot assays. The proliferation was assessed by cell count kit 8 and colony-forming assays. The apoptotic cells were determined by flow cytometry assay. The migration and invasion were analyzed by transwell assay. Colorectal cancer cells were co-cultured with peripheral blood mononuclear cells or cytokine-induced killer cells to assess immune response. The interaction relationships among circ-KRT6C, miR-485-3p, and PDL1 were examined by dual-luciferase reporter assay. The effects of circ-KRT6C inhibition *in vivo* was analyzed by an animal experiment. **Results:** Circ-KRT6C was overexpressed in colorectal cancer tissues and cells, and its level was associated with overall survival time of colorectal cancer patients. The suppression of circ-KRT6C suppressed growth, migration, invasion, and immune escape while stimulated apoptosis in colorectal cancer cells, which was abolished by shortage of miR-485-3p. In addition, overexpression of miR-485-3p repressed malignant progression and immune evasion of colorectal cancer by targeting PDL1, implying that PDL1 was a functional target of miR-485-3p. A xenograft experiment also suggested that circ-KRT6C inhibition could repress tumor growth *in vivo*. **Conclusion:** Circ-KRT6C could increase PDL1 expression by functioning as a miR-485-3p sponge, which promoted malignant progression and immune evasion of colorectal cancer cells. **Keywords:** circ-KRT6C, miR-485-3p, PDL1, colorectal cancer

**Significance statement**

Circ-KRT6C could increase PDL1 expression by functioning as a miR-485-3p sponge, which promoted malignant progression and immune evasion of colorectal cancer.
Introduction

Colorectal cancer is a common gastrointestinal malignancy, occupying the fourth primary reason of tumor-associated mortality all over the world (Marmol, Sanchez-de-Diego et al., 2017; Bray, Ferlay et al., 2018). Despite the progress in surgery and chemo/radiotherapy, postoperative metastasis and recurrence remain common challenges for terminal colorectal cancer patients (Modest, Pant et al., 2019). The pathogenesis of colorectal cancer is multi-factorial and complex processes, thus more attentions should be paid to clarify pathogenesis of colorectal cancer (Fearon, 2011).

Circular RNAs (circRNAs) are a group of RNAs that have a covalently closed loop structure (Salzman, 2016). Recently, it has been reported that circRNAs exerted multiple functions in the development of human diseases, including colorectal cancer (Tian, Xu et al., 2019). Circ-KRT6C (hsa_circ_0026416) is transcribed from keratin 6C (KRT6C) gene and located on chr12:52,863,194-52,865,516. However, the precise role of circ-KRT6C was unclear in colorectal cancer. The functional effects of circ-KRT6C was investigated in colorectal cancer progression.

MicroRNAs (miRNAs), small (18-25 nucleotides) and regulatory non-coding RNAs, which have been found to involve in regulating gene expression at the post-transcriptional level by targeting 3’untranslational region (UTR) of mRNAs (Lee & Dutta, 2009). Importantly, the downregulation of miR-485-3p was found in glioblastoma patients, suggesting a promising prognostic target for glioblastoma (Wang, Zhang et al., 2017). Similarly, the cancer-inhibitory role of miR-485-3p was evidenced in breast cancer (Lou, Xiao et al., 2016), renal cancer (Lai, Xin et al., 2020), and cervical cancer (Liu, Liu et al., 2020). However, the association relationships between circ-KRT6C and miR-485-3p have not been fully elucidated yet in colorectal cancer.

In addition, Programmed cell death protein 1 (PD-1) mainly expressed in immune cells, including CD4 T cells, CD8 T cells, while programmed cell death receptor ligand 1 (PDL1) is the ligand of PD-1. Previous reports suggested that PDL1
was used as a diagnosis biomarker and immune-checkpoint for human cancers (Akinleye & Rasool, 2019). Interestingly, PDL1 also expressed in cancer cells, and the upregulation of PDL1 could avoid anti-tumor immune responses through induction T-cell apoptosis and exhaustion by interacting with PD-1 on immune cells (Zheng, Fang et al., 2019). Importantly, immune evasion could help carcinogenesis and cancer progression (Vinay, Ryan et al., 2015). Therefore, the investigation targeting the PDL1 pathway was necessary for anti-tumor study.

Here we hypothesized that circ-KRT6C was involved in the immunosuppressive microenvironment and malignant progression in colorectal cancer via regulation of miR-485-3p and PDL1.

Materials and methods

Tissue specimens

All tissue specimens (tumorous and neighboring healthy tissues) were collected from colorectal cancer patients (N=26) at the Sixth Affiliated Hospital of Sun Yat-sen University. The removed specimens were instantly frozen in liquid nitrogen and then maintained in -80°C. All of the patients had provided the written informed consents before surgical procedure, and this research was ratified by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. Furthermore, 26 colorectal cancer patients were assigned to high (n=13) and low (n=13) expression groups in the light of median expression level of circ-KRT6C.

Cell lines and cell culture

The human normal intestinal epithelial cells (NCM460 cells) and colorectal cancer cell lines (Lovo, SW480, SW620, and HCT-116 cells) were purchased from the Nanjing Key Gen Biotech (Nanjing, China) and grown in RPMI 1640 medium (Wisent, Shanghai, China) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) under standard culture conditions (5% CO₂, 37°C).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted by Trizol (Thermo Fisher Scientific) following the manufacture’s protocol. After that, 5 μg of the RNA was reversely transcribed cDNA
by Transcription kit (Thermo Fisher Scientific). Quantification of RNA was conducted by SYBR Green Master Mix (CapitalBio, Beijing, China) on the IQTM5 Multicolour Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The expression levels of RNAs were standardized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 using comparative threshold cycle (Ct) method. The primer were showed: circ-KRT6C, 5’-GATCGCCACCTACCGCAAG-3’ (up) and 5’-TCCATGGATAGCACCACGGATG-3’ (down); KRT6C, 5’-CTTCCCTGCTCTCCGAGGTA-3’ (up) and 5’-GTGCTCAGATGGGGGCAGGTAT-3’ (down); miR-485-3p, 5’-GCCGAGGTCATACACGGCT-3’ (up) and 5’-CAGTGCAGGGTCCGAGGTAT-3’ (down); PDL1, 5’-GTTTAATGTCTTTTATATTAC-3’ (up) and 5’-CAGCATCCTGCAATTTTCAACA-3’ (down); U6, 5’-ATCCTTACGCACCCAGTCCA-3’ (up) and 5’-GAACGCTTCACGAATTTGCGC-3’ (down); and GAPDH, 5’-ACAGTCAGCCGCATCTTTCTCAG-3’ (up) and 5’-GACAAGCTTCCCGTTCAGCAG-3’ (down).

RNase R treatment and subcellular fractionation

For RNase R degradation experiments, total RNA (10 μg) was treated with 40 U RNase R (Thermo Fisher Scientific) for 1 h at 37°C. In addition, the cytoplasm and nuclear fraction RNA of colorectal cancer cells were isolated via the RNeasy Midi Kit (Qiagen, Hilden, Germany) and then subjected to RT-qPCR analysis.

Cell transfection

Short interfering RNA (siRNA) objecting circ-KRT6C (si-circ-KRT6C) and scrambled control (si-NC), circ-KRT6C-overexpressed vector (circ-KRT6C) and its control (pcDNA), and PDL1-overexpression vector (PDL1), and scrambled control (pcDNA) were provided by Geenseed Biotech (Beijing, China). MiR-485-3p mimic (miR-485-3p), miR-NC, miR-485-3p inhibitor (anti-miR-485-3p), and anti-miR-NC were synthesized by Biossci Company (Wuhan, China). SW620 and HCT-116 cells were sowed into the 6-well plate (1×10^6 cells/well), and then 2 μg of vectors, 80 nM of miRNA mimic, or 100 nM of miRNA inhibitor was transfected into colorectal
cancer by lipofectamine 2000 (CapitalBio) under the recommended protocol. Then SW620 and HCT-116 cells were collected at 48 h after transfection for subsequent experiments.

**Cell count kit 8 (CCK8) and colony formation assays**

The transfected SW620 and HCT-116 cells were seeded into the 96-well plate (3000 cells/well). After incubation for 48 h, 20 μL of CCK8 solution (Dojindo, Tokyo, Japan) was added to SW620 and HCT-116 cells and then incubated at 37°C for 4 h. A microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) was used to assess absorbance at wavelength of 450 nm each well. For colony formation experiments, stably transfected SW620 and HCT-116 cells were planted into the 24-well plate (600 cells per well) and then incubated at 37°C for 14 d. Eventually, cells were fastened and then dyed by crystal violet (Invitrogen, Carlsbad, CA, USA) for 20 min, and the colonies were assessed on the microscope (100× amplification; Eclipse TS100, Nikon, Tokyo, Japan).

**Cell apoptosis assay**

Colorectal cancer cells (SW620 and HCT-116 cells) were harvested by trypsin and then incubated with staining buffer containing Annexin V labeled with fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BestBio, Shanghai, China). After a 30 min incubation in dark condition, SW620 and HCT-116 cells were collected with centrifugation, following by measurement of cell apoptosis under the flow cytometry (Thermo Fisher Scientific).

**Transwell assay**

The 24-well transwell chamber (Corning, Franklin Lakes, NJ, USA) was used for migration assay. SW620 and HCT-116 cells were re-suspended in serum-free medium (1×10^5 cells/mL) and then seeded into the upper apical chamber, and the lower chamber was filled with complete medium (serum concentration: 10%) as nutrients. After 24 h incubation, SW620 and HCT-116 cells passed membranes were dyed by crystal violet (Invitrogen) and then assessed under a microscope (100× amplification; Eclipse TS100, Nikon). Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA) was used for invasion assay.
Enzyme linked immunosorbent assay (ELISA) and cytotoxicity activity analysis

The activated peripheral blood mononuclear cells (PBMCs) were provided from Nanjing Key Gen Biotech. Phytohaemagglutinin (PHA; Invitrogen) induced PBMCs were co-cultured with SW620 and HCT-116 cells in a humidified incubator for 24 h. The content of Interferon γ (IFN-γ) and tumor necrosis factor-α (TNF-α) was determined with the IFN-γ ELISA kit and TNF-α ELISA kit (Invitrogen) in the light of manufacturer’s instructions. For cytotoxicity activity analysis, the target cells (SW620 and HCT-116 cells) and effector cells (cytokine-induced killer cells; Nanjing Key Gen Biotech) were co-cultured in incubator at ratios of 1:15 for 24 h. The CCK8 assay was carried out to assess cell viability as above description, with only incubate target cells as control group.

Dual-luciferase reporter assay

Circinteractome (https://circinteractome.irp.nia.nih.gov/) and StarBase (http://starbase.sysu.edu.cn/) were used to predict targets of circ-KRT6C and miR-485-3p, respectively. For the luciferase assay, the sequences of circ-KRT6C containing miR-485-3p binding sequences were cloned into the pGL3-Report luciferase vector (Realgene, Nanjing, China) to establish WT-circ-KRT6C luciferase vector, with MUT-circ-KRT6C as control. Similarly, WT-PDL1 3’UTR and MUT-PDL1 3’UTR luciferase vectors were established. SW620 and HCT-116 cells were planted into the 6-well plates (1×10⁶ cells/well) and then co-transfected with plasmids and miR-485-3p mimic or control by Lipofectamine 2000 (CapitalBio). The Dual-Luciferase Assay Kit (Thermo Fisher Scientific) was used to measure luciferase activity in SW620 and HCT-116 cells after 24 h of transfection.

Western blot assay

Total proteins isolated from colorectal cancer cells or tissues were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes (Bio-Rad). After blocking by 5% skim milk, membranes interacted with primary antibodies overnight at 4°C, including anti-PDL1 (#13684S; 1:2500 dilution; Cell Signaling Technology, Danvers, MA, USA) and anti-β-actin (#4970S; 1:2500 dilution; Cell Signaling Technology). Subsequently, the
blots were interacted with secondary antibody (#7074S; 1:3500 dilution; Cell Signaling Technology) for 2 h and then visualized by Western Blotting Detection Kit (Solarbio, Beijing, China).

**In vivo experiment**

Animal experiment was performed with the approval from the Institutional Animal Care and Use Committee of the Sixth Affiliated Hospital of Sun Yat-sen University. BALB/c nude mice (Vital River Laboratory, Beijing, China) were maintained under specific pathogen-free conditions and fed according to the institutional guide. HCT-116 cells stably transfected with sh-circ-KRT6C (Geenseed Biotech) were hypodermically injected into the left axilla of nude mice (N=6; 5x10^6 cells per mouse). Tumor volume was assessed using the following formula: volume=1/2 (length×width^2).

**Statistical analysis**

Quantitative data were shown as mean ± standard deviation. Differences among groups were analyzed by Student’s t-test (two groups were compared) or one-way analysis of variance (>2 groups were compared) with multiple comparisons with post hoc Bonferroni test (SPSS 21.0; IBM, Somers, NY, USA). p<0.05 was considered significant difference. Pearson’s correlation coefficient analysis was used to analyze the correlations of two variables.

**Results**

**Circ-KRT6C was upregulated in colorectal cancer tissues and cells**

Initially, we measure the expression level of circ-KRT6C in colorectal cancer in colorectal cancer tissues by RT-qPCR. Analysis data of RT-qPCR suggested that circ-KRT6C was overexpressed in colorectal cancer tissues than that in control tissues; besides, colorectal cancer cells showed the high level of circ-KRT6C than control cells (Figure 1A-1B). Interestingly, Kaplan-Meier curves analysis revealed that colorectal cancer patients with low level of circ-KRT6C had longer survival times than colorectal cancer patients with high level of circ-KRT6C, suggesting the key
roles of circ-KRT6C in colorectal cancer (Figure 1C). Furthermore, circ-KRT6C was more capable of resistance to RNase R digestion than linear KRT6C; besides, circ-KRT6C was primarily expressed in the cytoplasm instead of nucleus in SW620 and HCT-116 cells (Figure 1D-1G). Therefore, the functional effects of circ-KRT6C were investigated in colorectal cancer.

**Knockdown of circ-KRT6C inhibited proliferation, migration, invasion, and immune escape while increased apoptosis in colorectal cancer cells**

To explore the functional roles of circ-KRT6C in colorectal cancer, SW620 and HCT-116 cells were transfected with si-circ-KRT6C to knock down the expression level of circ-KRT6C. Transfection with si-circ-KRT6C obviously inhibited the expression of circ-KRT6C in colorectal cancer cells (Figure 2A). In addition, the silencing of circ-KRT6C decreased the cell viability and colony number in SW620 and HCT-116 cells, suggesting that proliferation ability was inhibited by circ-KRT6C knockdown (Figure 2B-2C). Besides, circ-KRT6C knockdown could enhance the cell apoptosis of SW620 and HCT-116 cells (Figure 2D). The migration and invasion of colorectal cancer cells were all suppressed after inhibition of circ-KRT6C (Figure 2E-2F). Furthermore, cell supernatant levels of IFN-γ and TNF-α upregulated in PHA-stimulated PBMC was weakened by co-culturing with si-NC-transfected cells, while this immune suppressive effect was inhibited when co-cultured with si-circ-KRT6C-transfected cells (Figure 2G-2H). After co-culture with CIK cells, the cell survival rate of si-circ-KRT6C-transfected SW620 and HCT-116 cells was markedly lower than si-NC-transfected cells under the same conditions (Figure 2I). Therefore, circ-KRT6C played key roles in proliferation, apoptosis, migration, invasion, and immune escape of colorectal cancer cells.

**Circ-KRT6C could target miR-485-3p in colorectal cancer cells**

We speculated that circ-KRT6C could target miRNAs to regulate colorectal cancer progression, thus bioinformatics analysis (starBase) was performed. As presented in Figure 3A, circ-KRT6C had binding areas on miR-485-3p. Moreover, RT-qPCR assay suggested that miR-485-3p-transfected cells showed high level of miR-485-3p, while miR-485-3p was decreased in anti-miR-485-3p-transfected cells.
(Figure 3B). The upregulation of miR-485-3p dramatically repressed luciferase activity in WT-circ-KRT6C group, while no obvious effect was found in MUT-circ-KRT6C group (Figure 3C-3D). We also observed that miR-485-3p was downregulated in colorectal cancer tissues in comparison with negative groups (Figure 3E-3F). Besides, a negative correlation relationship between miR-485-3p and circ-KRT6C was found in colorectal cancer tissues (Figure 3G). The data of RT-qPCR revealed that circ-KRT6C was overexpressed in colorectal cancer cells transfected with circ-KRT6C than pCD5-ciR-transfected cells (Figure 3H). Importantly, inhibition of circ-KRT6C enhanced miR-485-3p expression in colorectal cancer cells, while overexpression of circ-KRT6C decreased miR-485-3p level (Figure 3I). Therefore, miR-485-3p expression was controlled by circ-KRT6C.

**Knockdown of miR-485-3p rescued circ-KRT6C silencing-induced effects on colorectal cancer cells**

To further investigate whether circ-KRT6C exerted functional role in colorectal cancer by targeting miR-485-3p, the rescue experiments were performed. The upregulation of miR-485-3p in si-circ-KRT6C-transfected cells was abolished by miR-485-3p inhibitor (Figure 4A). The results of CCK8 and colony-forming assays suggested that silencing of circ-KRT6C inhibited cell growth, which was overturned by inhibition of miR-485-3p (Figure 4B-4C). Additionally, knockdown of miR-485-3p weakened circ-KRT6C silencing-induced apoptosis in colorectal cancer cells (Figure 4D). Besides, the shortage of miR-485-3p increased the migration and invasion of si-circ-KRT6C-trated colorectal cancer cells (Figure 4E-4F). The suppression of circ-KRT6C increased immune effects in SW620 and HCT-116 cells by increasing IFN-γ and TNF-α levels, which was abolished by silencing of miR-485-3p (Figure 4G-4H). When co-culture with CIK cells, the cell viability inhibition caused by circ-KRT6C inhibition were overturned by miR-485-3p inhibitor in SW620 and HCT-116 cells (Figure 4I). These data suggested that circ-KRT6C exerted its role in colorectal cancer by targeting miR-485-3p.

**PDL1 was a functional target of miR-485-3p in colorectal cancer cells**

To figure out the target mRNA of miR-485-3p, bioinformatics analysis was
conducted using starBase. MiR-485-3p was documented to have binding regions on 3’UTR of PDL1 mRNA (Figure 5A). The overexpression of miR-485-3p effectively reduced the luciferase activity of WT-PDL1 3’UTR group, while MUT-PDL1 3’UTR group was not affected by miR-485-3p overexpression (Figure 5B-5C). The upregulation of PDL1 was found in colorectal cancer tissues and cells compared with matched controls (Figure 5D-5F). A negative correlation relationship between PDL1 and miR-485-3p was confirmed in colorectal cancer tissues (Figure 5G). In addition, overexpression of miR-485-3p decreased PDL1 expression, while inhibition of miR-485-3p upregulated PDL1 expression in SW620 and HCT-116 cells (Figure 5H). Therefore, PDL1 was a downstream gene of miR-485-3p in colorectal cancer cells. The increase of miR-485-3p inhibited proliferation, migration, invasion, and immune escape while induced apoptosis in colorectal cancer cells by targeting PDL1

To further investigate the association relationship between miR-485-3p and PDL1, functional experiments were performed. Transfection with PDL1 rescued miR-485-3p-induced the downregulation of PDL1 in colorectal cancer cells (Figure 6A). The proliferation suppression in miR-485-3p overexpression-cells was abolished by upregulation of PDL1 (Figure 6B-6C). Additionally, the data of flow cytometry assay displayed the miR-485-3p upregulation increased cell apoptosis, which was overturned by upregulation of PDL1 (Figure 6D). Transwell assay suggested that migration and invasion were inhibited by miR-485-3p overexpression, and these effects were rescued by overexpression of PDL1 in SW620 and HCT-116 cells (Figure 6E-6F). Moreover, the IFN-γ and TNF-α levels in cell supernatant was upregulated in miR-485-3p-transfected cells co-cultured with PBMC compared with control, while this effect was counteracted by overexpression of PDL1 (Figure 6G-6H). The cell survival rate of miR-485-3p-transfected SW620 and HCT-116 cells was obviously lower than control cells, which was overturned by upregulation of PDL1 (Figure 6I). Conclusively, miR-485-3p/PDL1 axis played key roles in proliferation, apoptosis, migration, invasion, and immune escape of colorectal cancer cells.
Circ-KRT6C regulated PDL1 expression by targeting miR-485-3p

As shown in Figure 7A-7B, the inhibition of circ-KRT6C decreased the expression of PDL1 in colorectal cancer cells, which was abolished by silencing of miR-485-3p. Conclusively, circ-KRT6C/miR-485-3p/PDL1 played important role in colorectal cancer progression.

Circ-KRT6C regulated tumor growth in vivo

The function roles of circ-KRT6C silencing in vivo were analyzed by a xenograft formation experiment. The inhibition of circ-KRT6C obviously inhibited tumor growth rate and weight in vivo compared to sh-NC group (Figure 8A-8B). Circ-KRT6C and PDL1 were downregulated while miR-485-3p was upregulated in sh-circ-KRT6C group in comparison with negative group (Figure 8C-8D). Therefore, knockdown of circ-KRT6C repressed tumor growth by regulating miR-485-3p and PDL1.

Discussion

Conclusively, our findings suggested that circ-KRT6C was obviously increased in colorectal cancer tissues and cells compared with negative groups. Mechanistically, circ-KRT6C stimulated immune evasion and pathological process of colorectal cancer by functioning as a miR-485-3p sponge to upregulate PDL1, providing a new diagnosis target for colorectal cancer patients.

CircRNAs usually play important roles in carcinogenesis as miRNA sponges for regulation of miRNA-mRNA network (Yuan, Peng et al., 2019). For example, circ-KRT6C promoted colorectal cancer progression by regulation of miR-346-Nuclear Factor I/B network, suggesting that circ-KRT6C was a novel carcinogenic circRNA in colorectal cancer (Liang, Shi et al., 2020). In general, a circRNAs can have many targets in biological processes. Therefore, we wondered whether circ-KRT6C could adsorb other miRNAs to regulate of colorectal cancer development. As we all known, the immune system played significant roles in elimination of cancer cells by working as cancer inhibitor, while immune evasion
could help carcinogenesis and cancer progression (de Visser, Eichten et al., 2006; Vinay, Ryan et al., 2015). As we expected, the inhibition of circ-KRT6C impeded cell growth, migration, invasion, and immune escape while induced apoptosis in colorectal cancer cells, accompanying the increases of cytokines including IFN-γ and TNF-α, depending on regulating miR-485-3p.

Consistently, the tumor-suppressive roles of miR-485-3p were reported in colorectal cancer; miR-485-3p could targetedly suppress the oncogene expression, such as target protein for Xenopus kinesin-like protein 2 in colorectal cancer cells (Taherdangkoo, Kazemi Nezhad et al., 2020). In this paper, miR-485-3p was found to have the binding sites for PDL1 and could bind to PDL1 in colorectal cancer cells. According to previous reports, miR-138-5p could target PDL1 to inhibit tumorigenesis in colorectal cancer and lung cancer (Zhao, Yu et al., 2016; Song, Li et al., 2020). Ashizawa et al. also revealed a regulatory mechanism of PDL1 expression that miR-148a-3p decreased PDL1 expression and suppressed immune evasion in colorectal cancer cells (Ashizawa, Okayama et al., 2019).

Moreover, previous evidence revealed that the posttranscriptional regulatory mechanisms of PDL1 were regulated by miRNAs. For example, PDL1 was regulated by miR-142-5p in pancreatic cancer, which enhance the anti-tumor immunity (Jia, Xi et al., 2017). Additionally, the high expression of PDL1 in cancer cells was involved in epithelial-to-mesenchymal transition and tumor cell evasion of the immune response (Noman, Janji et al., 2017; Jiang, Wang et al., 2019); on the contrary, the knockdown of PDL1 could restore immune suppression, leading activation of effector T cells and enhancement of anti-tumor immunity (Tang, Liu et al., 2019). Aside from these mechanisms, recent studies also focused on oncogenic pathway activation involved in PDL1 signal (Mimura, Kua et al., 2014; Casey, Tong et al., 2016). Therefore, PDL1 was an important anti-tumor therapeutic target due to it was expressed in most cancer cells. The drugs or antibodies targeting PDL1 were the promising therapeutic strategies in various types of cancer, which is being tested in clinical trials for multiple tumors (Hussain, Birtle et al., 2018; Wang, Zhou et al., 2019). Surely, a limited sample size, with only 26 samples were evaluated during the
Conclusively, our results suggested that a novel mechanism by which circ-KRT6C regulated miR-485-3p/PDL1 axis, and knockdown of circ-KRT6C could inhibit malignant progression and immune evasion of colorectal cancer cells. Therefore, targeting the circ-KRT6C/miR-485-3p/PDL1 axis might be a new immunotherapy for colorectal cancer patients.

Conclusion

Conclusively, our results revealed that circ-KRT6C was an oncogene in colorectal cancer, and circ-KRT6C could promote malignant progression and immune evasion of colorectal cancer cells through miR-485-3p/PDL1 axis, providing the novel information for future developing new therapeutic strategies for colorectal cancer patients.

Authors’ contributions

Participated in research design: Zhipeng Jiang and Zehui Hou. Conducted experiments: Wei Liu and Zhuomin Yu. Contributed new reagents or analytic tools: Zhiqiang Liang. Wrote or contributed to the writing of the manuscript: Zhiqiang Liang.

Acknowledgment

None.

Disclosure of interest

The authors declare that they have no financial conflicts of interest.

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References


Figure legends

**Figure 1** The level of circ-KRT6C in colorectal cancer tissues and cells. (A-B) The level of circ-KRT6C was determined by RT-qPCR in colorectal cancer tissues and cells, along with in matched negative groups. (C) Kaplan-Meier assay was performed to assess the association between overall survival and circ-KRT6C level in colorectal cancer patients. (D-E) After treating with RNase R, the expression of circ-KRT6C and linear NRBP1 was measured by RT-qPCR. (F-G) The level of circ-KRT6C was quantified by RT-qPCR in cytoplasmic and nuclear RNA fraction. *$P < 0.05$, ***$P <0.001$, ****$P <0.0001$.

**Figure 2** Effects of circ-KRT6C inhibition on proliferation, apoptosis, migration, invasion, and immune escape of colorectal cancer cells. (A-I) SW620 and HCT-116 cells were transfected with si-circ-KRT6C or si-NC. (A) The knockdown efficiency of si-circ-KRT6C was assayed by RT-qPCR. (B-C) The cell viability and colony number were presented by CCK8 and colony-forming assays, respectively. (D) The apoptosis rate was measured by flow cytometry assay. (E-F) The migration and invasion of SW620 and HCT-116 cells were evaluated by transwell assay. (G-H) The production of IFN-$\gamma$ and TNF-α was detected by ELISA in cell supernatant. (I) The survival rate of SW620 and HCT-116 cells was assessed by cytotoxicity activity analysis by CCK8 assay. **$P < 0.01$, ***$P <0.001$, ****$P <0.0001$.

**Figure 3** MiR-485-3p was a direct target of circ-KRT6C. (A) The complementary sequences between miR-485-3p and circ-KRT6C were shown. (B) The level of miR-485-3p was calculated by RT-qPCR in SW620 and HCT-116 cells transfected with miR-NC, miR-485-3p, anti-miR-NC, or anti-miR-485-3p. (C-D) Dual-luciferase reporter assay was used to confirm the association between miR-485-3p and circ-KRT6C. (E-F) The expression of miR-485-3p was evaluated by RT-qPCR. (G) The correlation between miR-485-3p and circ-KRT6C was assessed by Pearson’s correlation analysis. (H) The level of circ-KRT6C was tested by RT-qPCR in SW620 and HCT-116 cells transfected with circ-KRT6C or pCD5-ciR. (I) RT-qPCR assay was used to test miR-485-3p level in SW620 and HCT-116 cells transfected with si-NC, si-circ-KRT6C, circ-KRT6C, or pCD5-ciR. **$P < 0.01$, ***$P <0.001$, ****$P <0.0001$.
Figure 4 Knockdown of miR-485-3p abolished circ-KRT6C silencing-induced effects on colorectal cancer cells. (A-I) SW620 and HCT-116 cells were transfected with si-NC, si-circ-KRT6C, si-circ-KRT6C+anti-miR-NC, or si-circ-KRT6C+anti-miR-485-3p. (A) The expression level of miR-485-3p was tested by RT-qPCR. (B-C) CCK8 and colony-forming assays were used to assess the cell proliferation ability of SW620 and HCT-116 cells. (D) Flow cytometry assay was performed to evaluate cell apoptosis. (E-F) Transwell assay was used to determine migration and invasion of SW620 and HCT-116 cells. (G-H) ELISA was conducted to test cell supernatant levels of IFN-γ and TNF-α. (I) The survival rate was shown by CCK8 analysis. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 5 PDL1 was a functional gene of miR-485-3p. (A) The binding regions between PDL1 and miR-485-3p were shown. (B-C) Dual-luciferase reporter assay was conducted in transfected SW620 and HCT-116 cells. (D-F) RT-qPCR and western blot assays were used to assess PDL1 levels in colorectal cancer tissues and cells. (G) The correlation relationship between PDL1 and miR-485-3p was analyzed. (H) The protein level of PDL1 was quantified by western blot assay in SW620 and HCT-116 cells transfected with miR-NC, miR-485-3p, anti-miR-NC, or anti-miR-485-3p. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 6 The upregulation of miR-485-3p-caused effects on colorectal cancer cells were overturned by upregulation of PDL1. (A-I) SW620 and HCT-116 cells were transfected with miR-NC, miR-485-3p, miR-485-3p+pcDNA, or miR-485-3p+PDL1. (A) The protein expression level of PDL1 was quantified by western blot assay. (B-C) The cell viability proliferation was analyzed by CCK8 and colony-forming assays. (D) The apoptotic cells were monitored by flow cytometry assay. (E-F) The migrated and invaded cells were assessed by transwell assay. (G-H) The productions of IFN-γ and TNF-α were measured by ELISA. (I) The cytotoxicity activity was assessed by CCK8 assay. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7 Circ-KRT6C acted as a miR-485-3p sponge to upregulate PDL1. (A-B) The levels of PDL1 were determined by RT-qPCR and western blot in SW620 and
HCT-116 cells transfected with si-NC, si-circ-KRT6C, si-circ-KRT6C+anti-miR-NC, or si-circ-KRT6C+anti-miR-485-3p. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

**Figure 8 Inhibition of circ-KRT6C suppressed tumor growth.** (A-B) The volume and weight of xenograft were presented. (C-D) The level of circ-KRT6C, miR-485-3p, and PDL1 were tested by RT-qPCR and western blot in dissected tumor tissues. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. 
Figure 1

(A) Relative circ-KRT6C expression in Normal and Tumor samples.

(B) Relative circ-KRT6C expression in HCT116, LoVo, SW660, SW660-hKRT6C, and HCT14.

(C) Overall survival distribution.

(D) Relative RNA expression of circ-KRT6C and Linear KRT6C in Mock and RNase R samples.


(F) Relative RNA levels in Cytoplasm and Nucleus for SW660.

(G) Relative RNA levels in Cytoplasm and Nucleus for HCT116.
Figure 2
Figure 3
Figure 4
Figure 6
Figure 7

A

Relative PDL1 mRNA expression

SW620

si-NC

si-circ-KRT6C

si-circ-KRT6C+anti-miR-NC

si-circ-KRT6C+anti-miR-485-3p

PDL1

GAPDH

B

Relative PDL1 mRNA expression

HCT-116

si-NC

si-circ-KRT6C

si-circ-KRT6C+anti-miR-NC

si-circ-KRT6C+anti-miR-485-3p

PDL1

GAPDH
Figure 8

(A) Graph showing tumor volume (mm^3) over days.

(B) Bar graph comparing tumor weight (mg) between sh-NC and sh-circ-KRT6C.

(C) Bar graph showing relative RNA expression of circ-KRT6C and miR-485-3p.

(D) Western blot showing relative PDL1 protein expression between sh-NC and sh-circ-KRT6C.