SIRT6 inhibits colorectal cancer stem cell proliferation by targeting CDC25A

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Abstract. Silent information regulator 6 (SIRT6) is broadly considered as a tumor suppressor due to its function in the suppression of oncogene expression. However, the role of SIRT6 in colorectal cancer stem cells (CSCs) remains uncharacterized. In the present study, it was demonstrated that SIRT6 expression was reduced in colorectal CSCs. Overexpression of SIRT6 in colorectal CSCs did not induce cell apoptosis. However, SIRT6 significantly inhibited cell proliferation, colony formation and induced G0/G1 phase arrest in colorectal CSCs. In addition, SIRT6 repressed the expression of cell division cycle 25A (CDC25A), an oncogenic phosphatase. Chromatin immunoprecipitation experiments indicated that SIRT6 directly bound to the CDC25A promoter and decreased the acetylation level of histone H3 lysine 9. Altogether, these data indicated that SIRT6 inhibits colorectal cancer stem cell proliferation by targeting CDC25A.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men (746,000 cases, 10.0% of the total) and the second most common in women (614,000 cases, 9.2% of the total) worldwide (1,2). Despite a good outcome of treatment for early CRC, the clinical outcome for advanced CRC is unsatisfactory (1,2). Therefore, elucidating the molecular mechanisms underlying CRC progression is critical for developing novel therapeutic strategies to improve the prognosis of patients with advanced CRC.

Increasing evidence indicates that tumors are hierarchically organized, and only a small fraction of cells possess tumorigenic growth potential; these are termed cancer stem cells (CSCs) (3). The existence of CSCs was demonstrated in CRC, and cluster of differentiation (CD)133 was identified as the

marker for colorectal CSCs (4,5). CD133⁺ tumor cells behave like stem-like cells, and thereby produce more differentiated tumor cells in CRC (4,5). Despite numerous previous studies on these undifferentiated cancer cells, the signature profile and regulation of colorectal CSCs remains largely unknown.

Sirtuins are NAD⁺-dependent deacetylases that have been implicated in a diverse range of biological processes and various diseases, including cancer (6). SIRT6, one of the sirtuins, is primarily localized in the nucleus of the cells (6). It deacetylates histones H3 at either lysine 9 (H3K9) or lysine 56 (H3K56) and represses its target gene expression (7,8). Through its deacetylation activity, SIRT6 regulates a variety of cellular processes including chromosome stability, inflammation, cell metabolism, apoptosis and senescence (7,8).

Previous studies have indicated that SIRT6 serves important roles in the development of cancer: SIRT6 expression is downregulated in a variety of types of cancer, including hepatic and breast cancer, and head and neck squamous cell carcinoma (9-11). Downregulation of SIRT6 is positively correlated with poor prognosis and low tumor-free survival rates in CRC and pancreatic ductal adenocarcinoma (12,13). Deletion of SIRT6 in mice increases the number, size and aggressiveness of tumors (12,13). SIRT6 was also demonstrated to regulate cancer cell metabolism and promote cancer cell apoptosis (12,14). These studies indicated that SIRT6 serves as a general tumor suppressor in human cancer. However, the exact function of SIRT6 in colorectal CSCs remains unknown.

Cell division cycle 25A (CDC25A) is a member of the CDC25 phosphatases family that regulates cell cycle progression by inhibiting phosphorylation of the cyclin-dependent kinases (CDKs) subunit, thereby activating cyclin-CDK complexes (15). Elevated expression of CDC25A has been observed in various types of human cancer, and is often associated with high grade tumors and poor prognosis (16). Overexpression of CDC25A leads to genomic instability and tumorigenesis, while downregulation of CDC25A inhibits cellular transformation and tumorigenesis (15-17). Concurrently, overexpression of CDC25A in cancer tissues is accompanied by disorders in the cell cycle (15-17), suggesting that CDC25A is involved in the development and progression of tumors by regulating the cell cycle.

The present study investigated the role of SIRT6 in colorectal CSCs and the underlying mechanism. The results

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revealed a suppressive role of SIRT6 in colorectal CSCs growth and provided a potential target for CRC treatment.

Materials and methods

Tissue samples. The CRC tissue specimens were obtained from 10 male and 2 female patients at the Linyi People's Hospital (Linyi, China) following surgical resection between September 2016 and December 2016. The age of the patients ranged between 38 and 83 years. The types of CRC were all adenocarcinoma. The Ethics Committee of the Linyi People's Hospital approved the present study and written informed consent was obtained from all patients.

Cell culture. CRC SW480 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.). Cells were cultured at 37°C in an atmosphere containing 5% CO₂ and 100% humidity.

Flow cytometry analysis. Allophycocyanin (APC)-conjugated CD133 antibodies (AC133 epitope; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used for colorectal CSCs identification. Briefly, SW480 cells were digested using trypsin (Thermo Fisher Scientific, Inc.). CRC tissue was digested using collagenase (1.5 mg/ml, Life Technologies; Thermo Fisher Scientific, Inc.) and hyaluronidase (20 mg/ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBS for 1 h. Cells were centrifuged at 700 x g for 10 min at room temperature. Single-cell suspensions were then incubated with APC-CD133 antibodies on ice for 1 h in the dark. The cells were then isolated using FACSARIA (BD Biosciences, San Jose, CA, USA) flow cytometer. CD133-positive cells were considered to be colorectal CSCs. The populations of CSCs and non-CSCs from SW480 cell lines were then re-analyzed on a flow cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed using FlowJo software (version 7.6.1; FlowJo LLC, Ashland, OR, USA).

Transfection. SW480 CSCs were transiently transfected with pcDNA3.1 vector (Thermo Fisher Scientific, Inc.) or plasmid expressing SIRT6 gene (SIRT6 gene was constructed to pcDNA3.1) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Cells were subjected to the following experiments 24 h after transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from CSC cells was extracted using TRIzol® Reagent (Life Technologies; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Reverse transcription was performed at 37°C for 1 h and then inactivated at 70°C for 15 min according to the manufacturer's protocol (Life Technologies; Thermo Fisher Scientific, Inc.). qPCR assays were performed using the SYBR GREEN PCR Mix (Takara Bio Inc., Otsu, Japan) following the manufacturer's protocol. Briefly, PCR mixture were heated at 95°C for 30 sec and then subjected

to 40 cycles. For each cycle, the PCR mixture were first heated at 95°C for 5 sec and then amplified at 60°C for 30 sec. The expression levels of SIRT6 and CDC25A were normalized to the expression level of β -actin (ACTB). Primers 5'-CAAGTGTAAGACGCAGTACG-3' (forward) and 5'-GATGGTGTCCCTCAGCTCTC-3' (reverse) were used for SIRT6 amplification. Primers 5'-TGACATCTTTCAGCTCATCG-3' (forward) and 5'-CAGACAAAGTGGCTGTCACAG-3' (reverse) were used for CDC25A amplification. Primers 5'-TTGCGTTACACCCTTTCTTG-3' (forward) and 5'-CACCTTCACCGTTCCAGTTT-3' (reverse) were used for ACTB amplification. Data were analyzed using the 2^{- $\Delta\Delta C_q$} method (18).

Apoptosis assay. Colorectal CSCs were transfected with the empty vector or SIRT6-expressing plasmids for 48 h. Cells were then harvested and stained with Annexin V-fluorescein isothiocyanate (FITC; BioVision, Inc., Milpitas, CA, USA) for 5 min in the dark at room temperature. Cells were then analyzed by flow cytometry as aforementioned.

Cell proliferation assay. SW480 CSC cells were transfected with the empty vector or SIRT6-expressing plasmids. Following 24 h transfection, cells were seeded in 96-well plates at the density of 5x10³ cells/well. Cell number was detected using CCK-8 kit at 1, 2, 3, 4, and 5 days. Briefly, CCK-8 reagent was added to each well and the cells were incubated for 2 h at 37°C. The optical density OD at 450 nm was measured by using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Cell cycle analysis. For the cell cycle analysis, SW480 CSCs transfected with the empty vector or SIRT6-expressing plasmids were fixed with 100% ice-cold ethanol for 10 min, stained with propidium iodide (5 μ M; BioVision, Inc.) for 5 min at room temperature and examined with a flow cytometer as aforementioned.

Colony formation assay. Cells were seeded in 6-well plates at the density of 1,000 cells/well and maintained in DMEM containing 10% FBS. After 2 weeks, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature, and stained with crystal violet (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. The colony number was counted in 10 random fields of view using a wide-field light microscope at x5 magnification (Eclipse TS100; Nikon Corporation, Tokyo, Japan).

Protein extraction and immunoblotting. The whole cell lysate was extracted using lysis buffer (100 mM Tris, pH 7.8, 1% Triton X-100, 150 mM NaCl, 0.5 mM EDTA), and cleared by centrifugation at 12,000 x g for 10 min at 4°C. The protein concentration was determined using the BCA (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 50 μ g of proteins were subjected to SDS-PAGE (12% gel) electrophoresis and transferred to a nitrocellulose membrane (Life Technologies; Thermo Fisher Scientific, Inc.). The membrane was blocked with 3% milk in TBST buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature. The membrane was then incubated with antibodies targeting SIRT6 (1:1,000; rabbit; 12486; Cell Signaling Technology, Inc., Danvers,

Figure 1. SIRT6 expression is downregulated in colorectal CSCs. (A) Flow cytometry analysis was performed to detect the CD133 expression in CSCs and non-CSCs from SW480 cell lines. (B) Flow cytometry analysis was performed to detect the CD44 expression in CSCs and non-CSCs from SW480 cell lines. (C) The mRNA level of SIRT6 in SW480 CSCs and non-CSCs was measured by quantitative polymerase chain reaction. SIRT6 expression was calculated using ACTB as the endogenous control and normalized to the non-CSCs group. (D) The protein level of SIRT6 in SW480 CSCs and non-CSCs was detected by immunoblotting. The lower band represents the major isoform. (E) Scatter plot of SIRT6 expression in CSCs and non-CSCs isolated from colorectal cancer tissue (n=12). SIRT6 expression was calculated using ACTB as the endogenous control and normalized to the mean value of non-CSCs. ** $P<0.01$ (CSCs vs. non-CSCs). ACTB, β -actin; CD, cluster of differentiation; CSCs, cancer stem cells; SIRT6, Silent information regulator 6.

MA, USA), CDC25A (1:1,000; rabbit; 3652; Cell Signaling Technology) or β -actin (1:2,000; rabbit; 4970; Cell Signaling Technology) at 4°C overnight. Subsequent to 3 washes with Tris-buffered saline Tween-20 (TBST buffer), the membrane was then incubated with FITC-conjugated secondary antibodies (goat anti-rabbit; 926-32211; 1:5,000; LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h and detected with Odyssey Imaging System (version 2.1; LI-COR Biosciences).

Microarray analysis. Total RNA from control or SIRT6-expressing CSC cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was subjected to Agilent microarray analysis according to the manufacturer's protocol (KangCheng, Shanghai, China).

Chromatin immunoprecipitation (ChIP). ChIP assays were performed using the ChIP assay kit (17-371; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Briefly, SW480 CSCs were cross-linked with 1% formaldehyde for 10 min at 37°C. The samples were sonicated on ice in 30 sec intervals 6 times at the frequency of 20-60 kHz using Bioruptor Pico (Diagenode s.a., Seraing, Belgium) to obtain ~1,000 bp fragments. The lysates were cleared by centrifugation at 12,000 $\times$ g for 10 min at 4°C. The supernatant was diluted with ChIP dilution buffer and pre-cleared with salmon

sperm DNA/protein A-agarose (80 μ l/ChIP) at 4°C for 1 h. The lysates were incubated with 5 μ g anti-SIRT6, anti-acetylated histone H3 (Lysine9; 9649; Cell Signaling Technology), anti-acetylated histone H3 (Lysine56; ab21623; Abcam, Cambridge, MA, USA) or control IgGs overnight at 4°C. The immunocomplexes were then collected with protein A-agarose. The protein A-agarose beads were washed once with low salt buffer, once with high salt buffer, once with LiCl buffer and twice with Tris-EDTA buffer (Thermo Fisher Scientific, Inc.). The immunocomplexes were eluted and de-crosslinked at 65°C overnight. Following RNase digestion at 37°C for 30 min (Thermo Fisher Scientific, Inc.) and proteinase K digestion at 45°C for 1 h (Thermo Fisher Scientific, Inc.), the immunoprecipitated DNA was extracted and amplified by qPCR as aforementioned. Primers 5'-TGTAGGTCGGCTTGGTTTTC-3' (forward) and 5'-ATTAAATCCAAACAAACGTGG-3' (reverse) were used for CDC25A promoter amplification.

Statistical analysis. Statistical analysis was performed using SPSS (version 16.0; SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean $\pm$ standard deviation and evaluated with a double-sided Student's t-test. Statistical significance among >3 groups was determined using Turkey's post-hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference. Correlation analysis of SIRT6 and CDC25A expression in CSCs was performed using Pearson analysis.

Figure 2. SIRT6 does not affect colorectal CSCs apoptosis. SW480 CSCs were transfected with the empty vectors or SIRT6-expressing plasmids. (A) SIRT6 expression was detected by immunoblotting. (B) Cell apoptosis was assessed by Annexin V/propidium iodide staining. (C) Data from 3 independent experiments were analyzed and presented as mean $\pm$ standard deviation. N.S., not significant; Con, control; SIRT6, Silent information regulator 6; CSCs, cancer stem cells.

Results

SIRT6 expression is reduced in colorectal CSCs. To explore the role of SIRT6 in CRC stem cells, the CSCs population was separated from the SW480 cell line using the marker CD133. As expected, the isolated SW480 CSCs expressed high levels of CD133, while CD133 expression in the non-CSCs population was low (Fig. 1A). CD44, an additional CSCs marker, was also highly expressed in isolated SW480 CSCs as compared with non-CSCs (Fig. 1B). Next, the expression levels of SIRT6 in CSCs and non-CSCs were detected. The data indicated that SW480 CSCs expressed significantly decreased levels of SIRT6 compared with their corresponding non-CSCs population (Fig. 1C and D). Immunoblotting data also demonstrated that SIRT6 had several bands, which represented multiple isoforms (Fig. 1D).

To validate the reduced expression of SIRT6 in CSCs isolated from SW480 cells, the CSCs and non-CSCs were collected from the tumor tissues of patients with CRC. The data indicated that the mRNA level of SIRT6 in colorectal CSCs was significantly decreased compared with their corresponding non-CSCs (Fig. 1E). Taken together, the data above demonstrated decreased SIRT6 expression in colorectal CSCs.

SIRT6 does not affect cell apoptosis. SIRT6 was downregulated in colorectal CSCs; the effect of SIRT6 on colorectal CSC behavior was therefore investigated. As SIRT6 was previously demonstrated to induce cancer cell apoptosis (14), SIRT6-regulated apoptosis was first explored. The immunoblotting data suggested that SIRT6 protein was almost undetectable in SW480 CSCs; therefore, SIRT6 overexpression was then successfully induced (Fig. 2A). This was also represented at the mRNA level. SIRT6 mRNA level in the SIRT6 overexpression group increased 200-fold compared with the control group (data not shown). There was no difference in Annexin V staining observed between the control

and SIRT6-expressing groups, indicating that SIRT6 does not induce cell apoptosis in SW480 CSCs (Fig. 2B and C).

SIRT6 inhibits SW480 cell proliferation. Next, the effect of SIRT6 on SW480 CSCs proliferation was detected. The CCK-8 assay demonstrated that compared with the control group, the proliferation rate was significantly decreased in SIRT6-expressing cells (Fig. 3A), indicating that SIRT6 inhibits SW480 CSCs proliferation. Cell proliferation is tightly controlled by the cell cycle (19). Therefore, the cell cycle distribution was examined using flow cytometry. The data suggested that SIRT6 expression in SW480 CSCs increased the number of cells in G0/G1 phase (50 vs. 30%), while decreased the number of cells in S phase (29 vs. 48%; Fig. 3B and C), indicating that SIRT6 induces G0/G1 arrest in colorectal CSCs.

The colony formation assay indicated that the expression of SIRT6 in SW480 CSCs significantly decreased the colony number (Fig. 3D and E; 36 ± 8 vs. 64 ± 8), additionally supporting the growth inhibitory effect of SIRT6 on colorectal CSCs.

SIRT6 represses CDC25A expression in SW480 cells. SIRT6 represses its target gene expression (7,8). To identify the SIRT6 target genes in SW480 CSCs, SIRT6 was expressed and microarray analysis was performed. Using a 2-fold difference (upregulation and downregulation) as the cutoff, 120 downregulated genes were identified following SIRT6 overexpression (data not shown). CDC25A was among the top 10 significantly downregulated genes, and is closely associated with cell cycle and tumorigenesis (16). Therefore, CDC25A was selected for additional analysis. To confirm the effect of SIRT6 on CDC25A expression, independent overexpression experiments were performed. The data indicated that the mRNA level (Fig. 4A) and the protein level (Fig. 4B) of CDC25A was significantly decreased in SIRT6-expressing cells ($P < 0.01$). Furthermore, expression of SIRT6 increased the

Figure 3. SIRT6 inhibits the proliferation of colorectal CSCs. SW480 CSCs were transfected with the empty vectors or SIRT6-expressing plasmids. (A) The cell proliferation rate was assessed by CCK-8 assay at the indicated time. Data are presented as mean $\pm$ standard deviation of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ (Control vs. SIRT6 overexpression group). (B) Cell cycle distributions were analyzed by flow cytometry. (C) Cell cycle distributions from three independent experiments were analyzed. ** $P < 0.01$. (D) Colony formation was performed and (E) colony number was quantified. ** $P < 0.01$ (Control vs. SIRT6 overexpression group). SIRT6, Silent information regulator 6; CSCs, cancer stem cells; CSCs, cancer stem cells.

Figure 4. SIRT6 decreases CDC25A expression in colorectal CSCs. SW480 CSCs were transfected with empty vector or SIRT6-expressing plasmid. (A) The mRNA level of SIRT6 and CDC25A was detected by quantitative polymerase chain reaction. ** $P < 0.01$ (Control vs. SIRT6 overexpression group). (B) The protein level of CDK2, SIRT6 and CDC25A, and the phosphorylation of CDK2 at Thr14 were detected by immunoblotting. SIRT6, Silent information regulator 6; CDK2, cyclin-dependent kinase 2; CDC25A, cell division cycle 25A; p-CDK2, phosphorylated CDK2; CSCs, cancer stem cells.

phosphorylation level of cyclin-dependent kinase 2 (CDK2) at Thr14, a target of CDC25A, without affecting the protein level of CDK2 (Fig. 4B). These data indicated that SIRT6 inhibits the expression of CDC25A in SW480 CSCs.

SIRT6 decreases the H3K9 acetylation level at the promoter of CDC25A. To assess whether SIRT6 regulates CDC25A expression by directly binding to its promoter, ChIP was performed using SIRT6 antibodies. The results revealed that the level of precipitated CDC25A promoter was increased in the SIRT6 antibody group compared with the IgG group, demonstrating that SIRT6 binds to CDC25A promoter. Overexpression of SIRT6 significantly increased the binding level of SIRT6 with the CDC25A promoter (Fig. 5A).

SIRT6 deacetylates histones H3 at either lysine 9 (H3K9) or lysine 56 (H3K56) (7,8). To determine which lysine SIRT6 deacetylated at the CDC25A promoter region, SIRT6 was overexpressed and ChIP was performed using H3K9 acetylation or H3K56 acetylation antibodies. The data indicated that SIRT6 expression decreased H3K9 acetylation and H3K56 acetylation at the promoter region of the Lactate dehydrogenase B gene, which was demonstrated to be a SIRT6 target gene (12,13). However, SIRT6 expression only decreased the level of H3K9 acetylation, not H3K56 acetylation, at the CDC25A promoter region (Fig. 5B and C).

Finally, the expression levels of SIRT6 and CDC25A in CRC tissues were detected. The results suggested that SIRT6 expression was negatively correlated with CDC25A expression in CRC tissue ($r = 0.563$, $P = 0.005$; Fig. 5D).

Discussion

The tumor-suppressing role of SIRT6 in cancer progression has been identified previously: Firstly, SIRT6 expression is downregulated in various types of cancer (9-11); secondly,

Figure 5. SIRT6 decreases the H3K9 acetylation level at the promoter of CDC25A. (A) SW480 CSCs transfected with empty vectors or SIRT6-expressing plasmids were subjected to chromatin immunoprecipitation assays using anti-SIRT6 antibodies or control IgG. The promoter region as indicated were detected by qPCR. The anti-SIRT6 group was normalized to the IgG group. ** $P < 0.01$ (IgG vs. α SIRT6; Control vs. SIRT6 overexpression group). (B and C) SW480 CSCs transfected with empty vectors or SIRT6-expressing plasmids were subjected to chromatin immunoprecipitation assays using (B) Ac-H3K9 or (C) Ac-H3K56 antibodies. The promoter region as indicated were detected by qPCR. ** $P < 0.01$ (Control vs. SIRT6 overexpression). (D) Correlation analysis of SIRT6 and CDC25A expression in CSCs was performed using a Pearson analysis. SIRT6, Silent information regulator 6; IP, immunoprecipitation; α SIRT6, anti-SIRT6 antibody; CDC25A, cell division cycle 25A; LDHB, Lactate dehydrogenase B; qPCR, quantitative polymerase chain reaction; CSCs, cancer stem cells.

SIRT6 inhibits tumorigenesis and tumor development in mouse models (12,13). However, the oncogenic role of SIRT6 has also been described: Sociali *et al* identified that the inhibition of SIRT6 sensitizes pancreatic cancer cells to chemotherapeutics (20). In addition, SIRT6 was also demonstrated to prevent hepatocellular carcinoma cell apoptosis by suppressing B cell lymphoma 2-associated X protein expression (21). Therefore, the precise role of SIRT6 may be dependent on the various types of cancer or the specific stages and requires further investigation. The data of the present study indicated that SIRT6 expression was reduced in colorectal CSCs. Overexpression of SIRT6 inhibited colorectal CSCs proliferation and induced G0/G1 cell cycle arrest *in vitro*. All these data supported a suppressive role of SIRT6 in CRC.

SIRT6 may be regulated at transcriptional or post-transcriptional levels (22). Ubiquitin-mediated degradation is an important aspect for SIRT6 expression regulation in human cancer (22). Thirumurthi *et al* demonstrated that SIRT6 was phosphorylated at Ser (338) by the kinase AKT1, which mediated the ubiquitination of SIRT6 by Mouse double minute 2 homolog, targeting SIRT6 for protease-dependent degradation in breast cancer cell lines (22). Destabilization of SIRT6 thereby promotes tumorigenesis and trastuzumab resistance in breast cancer. The data of the present study suggested that the mRNA level of SIRT6 was decreased in colorectal CSCs, indicating an inhibition of SIRT6 transcription in colorectal CSCs. Exploring potential repressors of SIRT6 transcription in colorectal CSCs may provide insight on its expression regulation, and may identify potential targets for CRC therapy.

Due to the critical role of CDC25A in cell cycle regulation and cancer development, the protein level of CDC25A requires strict regulation (15-17). This may be achieved through multiple mechanisms, including regulating CDC25A expression at transcriptional, translational or post-translational levels (23). CDC25A protein degradation is regulated by a ubiquitin-proteasome system, and stabilization of CDC25A is one of the major causes for the upregulation of this protein in cancer (24). Pereg *et al* (24) revealed that ubiquitin hydrolase Ubiquitin-specific peptidase 17-like family member 2 (Dub3) reduces CDC25A degradation by removing its ubiquitin chains, and by stabilizing CDC25A, Dub3 promotes oncogenic transformation in breast cancer. Activation of CDC25A transcription also induces the upregulation of this protein in cancer: Human papillomavirus type 16 E7 oncoprotein was demonstrated to upregulate CDC25A transcription by disruption of the E2F transcription factor 1-Retinoblastoma protein inhibitory complex (25). Tumor suppressors, conversely, repress CDC25A transcription. For example, tumor protein 53 was suggested to activate Activating transcription factor 3, resulting in its direct binding to the CDC25A promoter and inhibition of CDC25A transcription (26). The data of the present study demonstrated that overexpression of SIRT6 in colorectal CSCs resulted in significantly decreased expression of CDC25A. The ChIP experiments demonstrated that SIRT6 binds to CDC25A promoter and reduces H3K9 level, indicating that CDC25A expression is regulated through an epigenetic mechanism by SIRT6 in colorectal CSCs. Additional studies on SIRT6 regulation of CDC25A expression in the development of CRC will increase our understanding of the regulation of CDC25A expression in cancer.

To conclude, the present study identified that SIRT6 expression is reduced in colorectal CSCs. Furthermore, it was demonstrated that SIRT6 inhibits cell proliferation and CDC25A expression in colorectal CSCs. These results indicate a suppressive role of SIRT6 in the development of CRC, and implicate its potential application in CRC therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WL and JL designed the experiments. WL, MW, HD, XS and TZ performed the experiments and analysis.

Ethics approval and consent to participate

The Ethics Committee of the Linyi People's Hospital approved the present study and written informed consent was obtained from all patients.

Consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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