

Downregulation of *SMC1A* inhibits growth and increases apoptosis and chemosensitivity of colorectal cancer cells

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Abstract

Objective: The structural maintenance of chromosomes (SMC) IA protein is a component of the cohesin multiprotein complex that is essential for sister chromatid cohesion. *SMC1A* gene mutations have been reported in colorectal cancer. This study aimed to investigate the role of *SMC1A* gene expression in colorectal cancer *in vitro*.

Methods: *SMC1A* gene expression was silenced by lentivirus-mediated infection with small interfering RNA (siRNA) in the human colorectal cancer cell line HT-29. Cell proliferation rates, *SMC1A* mRNA and protein levels, apoptosis and chemosensitivity to oxaliplatin were evaluated using routine *in vitro* assays, real-time polymerase chain reaction, Western blotting and flow cytometry.

Results: Knockdown of *SMC1A* protein and mRNA levels resulted in the inhibition of cell proliferation, an increased rate of apoptosis and enhanced chemosensitivity to oxaliplatin in HT-29 cells.

Conclusions: The findings of this study suggest that *SMC1A* plays an oncogenic role in colorectal cancer and that it might be a promising target for colorectal cancer therapy.

Keywords

Structural maintenance of chromosomes IA protein, *SMC1A*, colorectal cancer, chemosensitivity, oxaliplatin

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Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related mortality worldwide, especially in Western countries.¹ CRC arises through several mechanisms.² Microsatellite instability caused by a mismatch repair defect is common in familial CRC.³ CpG island hypermethylation is another mechanism of gene inactivation in CRC.⁴

Recent evidence suggests that the cohesin multiprotein complex is implicated in several diseases.^{5,6} The cohesin multiprotein complex includes four major subunits: structural maintenance of chromosomes (SMC) 1A; SMC3; sister chromatid cohesion (SCC) protein 1; SCC3.⁷ The cohesin multiprotein complex plays an important role in the regulation of transcription and development.⁸ *SMC1A* is an X-linked gene that could escape X-inactivation in humans, but is subject to X-inactivation in the mouse.⁹ Several mutations have been identified in the *SMC1A* gene, all of which are missense or small deletion mutations.^{10,11} Although *SMC1A* mutations have been reported in CRC,⁶ the role of *SMC1A* in CRC remains unclear.

Oxaliplatin is a commonly used platinum-based compound for the treatment of CRC.¹² The nucleotide excision repair pathway is mainly involved in the processing of oxaliplatin.¹³ *SMC1A* regulates DNA damage-induced cell-cycle checkpoint and DNA repair.¹⁴ Therefore, it is speculated that *SMC1A* may regulate oxaliplatin sensitivity in CRC. In this study, a loss-of-function approach was employed to silence *SMC1A* gene expression using small interfering RNA (siRNA) in the human CRC cell line; HT-29; the effects of *SMC1A* gene knockdown on cell growth, cell-cycle progression, apoptosis and chemosensitivity to oxaliplatin were examined.

Materials and methods

Cell culture

Both HEK293T cells and human CRC HT-29 cells were purchased from the American

Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (both from Gibco, Carlsbad, CA, USA) at 37°C in a humidified atmosphere containing 5% carbon dioxide.

SMC1A siRNA infection

Either the *SMC1A* siRNA or the negative control siRNA were synthesized (Gene Chem, Shanghai, China): *SMC1A* siRNA sequence: 5'-taGGAGGTTCTTCTGAGTACA-3'; negative control siRNA sequence: 5'-GGAGGTTCTTCTGAGTACA-3'. They were inserted into the pGCSIL-GFP vector (GeneChem) using *AgeI* and *EcoRI* restriction sites, and then transfected into HEK293T cells together with lentiviral helper plasmid pHelper1.0 and pHelper2.0 using Lipofectamine[®] 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions, to generate lentiviruses. Recombinant lentivirus containing either the siRNA targeting *SMC1A* or the negative control siRNA were prepared and titrated to $\sim 10^7$ transfection units/ml. HT-29 cells (1×10^4 cells/well) were seeded into a 24-well plate. After 24 h, the HT-29 cells were incubated with viral stocks at a multiplicity of infection level of 5 for 1 h. Confirmation that the lentiviral infection had been successful was demonstrated by observing the presence of green fluorescent protein within the HT-29 cells using a fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan).

Real-time PCR

To evaluate whether *SMC1A* siRNA could inhibit the production of *SMC1A* mRNA in HT-29 cells, real-time PCR analysis was undertaken to detect *SMC1A* mRNA levels in HT-29 cells infected with *SMC1A* siRNA lentivirus or negative control lentivirus. Total RNA was extracted from 1×10^5

HT-29 cells using TRIzol[®] reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2 µg) was reverse transcribed using a Moloney Murine Leukemia Virus Reverse Transcriptase kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The resulting cDNA was used for real-time polymerase chain reaction (PCR) using the SYBR[®] Green Master PCR Mix (Applied Biosystems, Foster City, CA, USA) in triplicates using a TP800 Thermal Cycler DiceTM Real Time System (TaKaRa, Dalian, China). The primer sequences for real-time PCR for SMC1A were 5'-GGAGCAGCAGCAGATTGAG-3' (sense) and 5'-TCTCTTCTTCCATCCGTTCTTC-3' (antisense). Primers for the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5'-TGACTTCAACAGCGACACCCA-3' (sense) and 5'-ACCCTGTTGCTGTAG CCAA-3' (antisense). All primers were synthesized by GeneChem. The cycling programme involved preliminary denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 45 s, followed by a final elongation step at 72°C for 5 min. The relative mRNA levels of SMC1A were compared with those of GAPDH and calculated using the $2^{-\Delta\Delta C_T}$ method. The C_T value used for these calculations was the mean of the triplicate for each reaction.

Western blot analysis

Total protein was extracted from 1×10^6 HT-29 cells using ice-cold RIPA Lysis and Extraction Buffer (ThermoFisher Scientific, Waltham, MA, USA) and quantified using a Pierce BCA protein assay reagent kit (Pierce Biotechnology, Rockford, IL, USA). The proteins (50 µg per lane) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis at 150 V for 90 min. The proteins were then transferred to polyvinylidene fluoride membranes

(Millipore, Billerica, MA, USA) using electroblot apparatus at 80 V for 1 h (Bio-Rad, Hercules, CA, USA). The membranes were incubated in blocking solution consisting of Tris-buffered saline-Tween 20 (TBST; pH 7.5; 20 mmol/l Tris-HCl, 150 mmol/l sodium chloride, 0.1% Tween-20) containing 5% nonfat milk at room temperature for 1 h. The membranes were then incubated with rabbit antihuman antibody for SMC1A or β -actin (dilution 1:1000; Cell Signaling Technology[®], Danvers, MA, USA) at 4°C overnight. The membranes were then washed with TBST (pH 7.5) three times and incubated with horseradish peroxidase-conjugated goat antirabbit secondary antibody (dilution 1:4000, Cell Signaling Technology[®]) for 1 h at room temperature. The membranes were developed using an enhanced chemiluminescence reagent kit (Pierce Biotechnology) and exposed to X-radiography film. Immunoblots were scanned using a densitometer (Bio-Rad) and analysed using Quantity One[®] software, version 4.6.2 (Bio-Rad).

Cell proliferation assay

To examine effect of SMC1A siRNA on HT-29 cell proliferation, HT-29 cells infected with SMC1A siRNA (SMC1A-knockdown [KD]) or control siRNA (control) were plated into 96-well plates at 2×10^3 cells/well and cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide. Cells were counted and imaged using an ArrayScanTM XTI High Content System reader (ThermoFisher Scientific) every day at fixed timepoints. Data from three independent experiments were analysed.

BrdU incorporation assay

The SMC1A-KD and control cells were seeded into 96-well plates at 2×10^3 cells/well and cultured for 24 h or 96 h at 37°C in

a humidified atmosphere containing 5% carbon dioxide. They were then incubated with a final concentration of 10 μ M 5-bromo-2-deoxyuridine (BrdU; BD Biosciences, San Jose, CA, USA) for 4 h. Cells were then fixed in 1% paraformaldehyde for 15 min and labelled with peroxidase-conjugated antiBrdU antibody (dilution 1:1000; Millipore) at 37°C for 1 h. Next, peroxidase substrate (50 μ mol/l tetramethylbenzidine) was added and incubated at room temperature for 15 min. The absorbance values were measured at 450 nm using a microplate reader (BD Biosciences).

Flow cytometric analysis of cell cycle

The effect of SMC1A knockdown on the cell cycle of HT-29 cells was determined using flow cytometry. For cell-cycle analysis, 1×10^5 SMC1A-KD and control cells were seeded into a 6 cm dish and cultured at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 24 h, 1×10^6 cells were harvested, washed twice with ice-cold 0.01 M phosphate buffered saline (PBS; pH 7.2), and fixed in 70% ethanol for 1 h at room temperature. Cells were washed twice with ice-cold 0.01 M PBS (pH 7.2) and incubated with 50 μ g/ml propidium iodide solution (Sigma-Aldrich, St Louis, MO, USA) and 100 μ g/ml RNase A (Fermentas, Glen Burnie, MD, USA) in 0.01 M PBS (pH 7.2) for 15 min at room temperature in the dark. The cell samples were then subjected to flow cytometric analysis using a BD FACSCalibur™ system (BD Biosciences). The percentage of HT-29 cells in each phase of the cell cycle was calculated.

Cell apoptosis assay

Approximately 1×10^7 SMC1A-KD and control cells were harvested and stained using an apoptosis detection kit (Annexin V Apoptosis Detection Kit APC; eBioscience, San Diego, CA, USA) according to the

manufacturer's instructions. Cell samples were then subjected to flow cytometric analysis using a BD FACSCalibur™ system, using protocols provided the manufacturer. The percentage of HT-29 cells that were apoptotic was calculated.

Chemosensitivity assay

To investigate whether the sensitivity of HT-29 cells to oxaliplatin was affected by SMC1A knockdown, the inhibitory effect of oxaliplatin on HT-29 cell proliferation was examined. Untreated HT-29, SMC1A-KD and control cells were exposed to different concentrations of oxaliplatin (Sigma-Aldrich) and the cell proliferation inhibitory rate was determined as follows. In brief, the cells were seeded into 96-well plates at 5×10^5 cells/well and cultured for 48 h at 37°C in a humidified atmosphere containing 5% carbon dioxide. Then, cells were exposed to various concentrations of oxaliplatin (1, 2, 4, 6, 8, 12 and 16 μ mol/l) for a further 48 h at 37°C. A CCK-8 assay was performed using a commercial kit (Dojindo Laboratories, Kumamoto, Japan) following the manufacturer's instructions and the absorbance at 450 nm was read using a microplate reader (Bio-Rad).

Statistical analyses

All statistical analyses were performed using the SPSS® statistical package, version 17.0 (SPSS Inc., Chicago, IL, USA) for Windows®. All data were presented as mean \pm SD. Differences between continuous variables were analysed using Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

Results

When real-time PCR analysis was undertaken to detect SMC1A mRNA levels in HT-29 cells infected with SMC1A siRNA

lentivirus or negative control lentivirus, under a fluorescence microscope, most of the infected cells were observed to be green. This indicated that the *SMC1A* siRNA lentivirus or negative control lentivirus had been efficiently delivered into the HT-29 cells (Figure 1A). The *SMC1A* mRNA level was significantly lower in HT-29 cells infected with *SMC1A* siRNA lentivirus (*SMC1A*-KD cells) than in HT-29 cells infected with negative control lentivirus (control cells) ($P < 0.05$) (Figure 1B).

Furthermore, Western blot analysis showed that the *SMC1A* protein level was lower in the *SMC1A*-KD cells than in control cells (Figure 1C). Taken together, these data suggested that the *SMC1A* siRNA was able to knockdown *SMC1A* mRNA and protein production in HT-29 cells, which was essential to know prior to undertaking the subsequent experiments.

In the cell proliferation assay, HT-29 cells treated with *SMC1A* siRNA showed a lower growth rate compared with those treated with negative control siRNA from day 2 to day 5 (Figure 2A). Results of the BrdU incorporation assay showed that the rate of DNA synthesis in HT-29 cells treated with *SMC1A* siRNA lentivirus was not affected on day 1 after infection. On day 4, DNA synthesis was significantly suppressed by 29.4% in HT-29 cells treated with *SMC1A* siRNA compared with those treated with control siRNA ($P < 0.05$) (Figure 2B).

Flow cytometry indicated that the number of HT-29 cells treated with *SMC1A* siRNA in the G₂/M phase was increased, while the number of cells in the S phase was significantly decreased, compared with HT-29 cells treated with control siRNA ($P < 0.05$) (Figure 3A). As shown in Figure 3B, the number of apoptotic cells was significantly increased in *SMC1A*-KD cells compared with control cells ($P < 0.05$).

The CCK-8 assay demonstrated that oxaliplatin showed stronger toxicity for *SMC1A*-KD cells ($IC_{50} 5.65 \pm 0.05 \mu\text{mol/l}$)

than for Control cells ($IC_{50} 7.40 \pm 0.02 \mu\text{mol/l}$) or untreated HT-29 cells ($IC_{50} 7.62 \pm 0.07 \mu\text{mol/l}$).

Discussion

In this present study, siRNA was employed to knockdown the expression of the *SMC1A* gene in the CRC cell line HT-29. Knockdown of the *SMC1A* gene inhibited proliferation and cell-cycle progression of HT-29 cells, while apoptosis and the sensitivity to oxaliplatin were enhanced. These present findings suggest that the *SMC1A* protein plays an oncogenic role in CRC cells and that *SMC1A* gene-targeted siRNA could be used for the gene therapy of CRC.

The cohesin multiprotein complex is formed by several subunits such as *SMC1A*, *SMC3*, *SCC1* and *SCC3*.⁷ *SMC* proteins contain globular N- and C-terminal domains with adenosine triphosphate (ATP)-binding Walker A and Walker B motifs.⁷ ATP binding promotes the association of the two intermolecular heads while ATP hydrolysis drives them apart.¹⁵ Mutations in the *Bacillus SMC* hinge domain could disrupt DNA binding and ATP hydrolysis.¹⁵ Therefore, *SMC1A* mutations would inhibit sister chromatid cohesion and disrupt cell division. Interestingly, *SMC1A* mutations have been reported in CRC, but whether *SMC1A* mutations are implicated in the tumourigenesis of CRC needs further investigation.

Oxaliplatin has demonstrated antitumour activity against CRC.¹⁶ However, one of the major obstacles for the application of oxaliplatin is intrinsic and acquired resistance.¹⁶ Oxaliplatin resistance is related to the abnormality in DNA repair and apoptosis.¹⁷ *SMC1A* is known to regulate DNA damage-induced cell-cycle checkpoint and DNA repair.¹⁴ In this present study, knockdown of *SMC1A* mRNA increased the sensitivity of HT-29 cells to oxaliplatin and this appeared to coincide with cell-cycle arrest and increased apoptosis, which were also induced by *SMC1A* gene

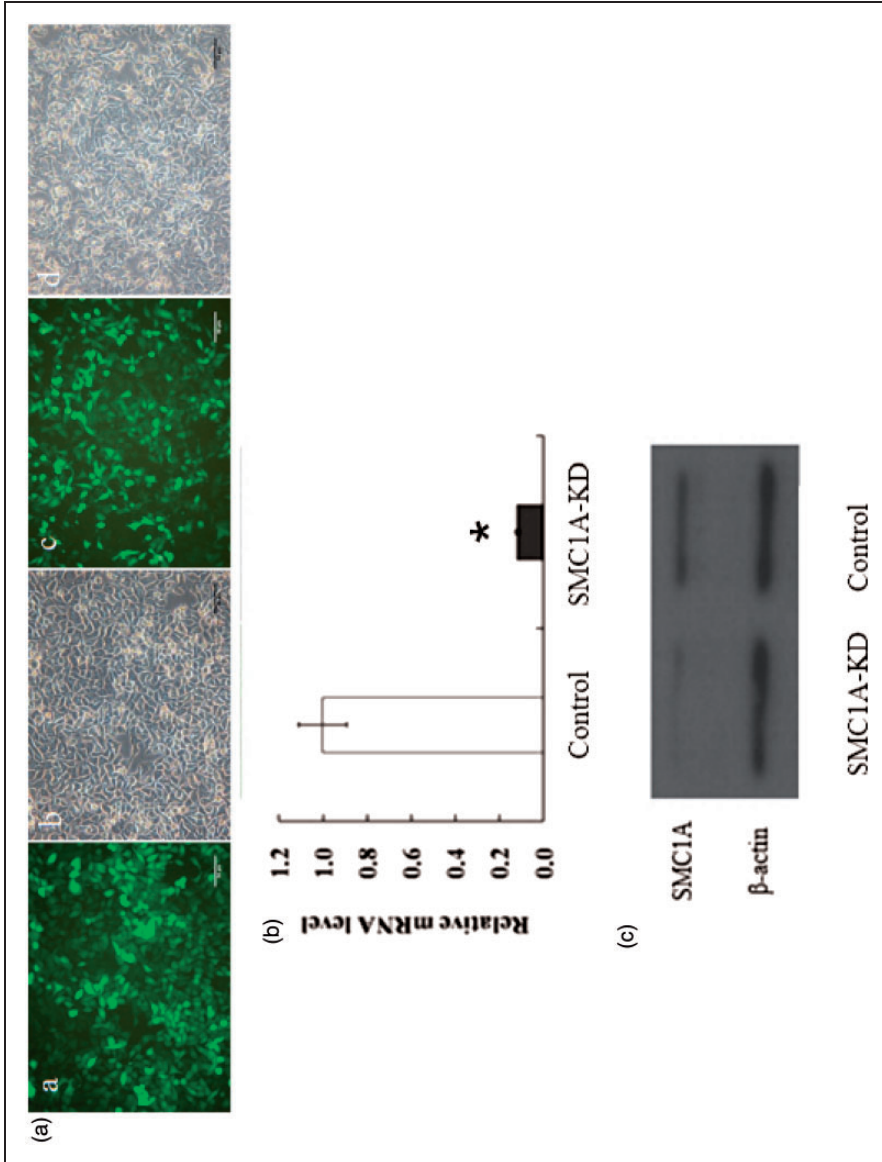


Figure 1. Knockdown of structural maintenance of chromosomes (SMC) 1A mRNA by lentivirus-mediated small interfering RNA (siRNA) in HT-29 cells. (a) Microscopic images of control siRNA lentivirus-infected HT-29 cells (Control) and SMC1A siRNA lentivirus-infected HT-29 cells (SMC1A-KD): a. Control cells under a fluorescence microscope; b. Control cells under a light microscope; c. SMC1A-KD cells under a fluorescence microscope; d. SMC1A-KD cells under a light microscope. Scale bar: 50 μ m. (b) Real-time polymerase chain reaction analysis of the SMC1A mRNA levels in Control and SMC1A-KD cells. Data presented as mean \pm SD ($n = 3$). * $P < 0.05$, Student's t -test. (c) Western blot analysis of SMC1A protein levels in Control and SMC1A-KD cells; Representative blot from three independent experiments that had similar results. β -actin was the loading control. The colour version of this figure is available at: <http://imr.sagepub.com>.

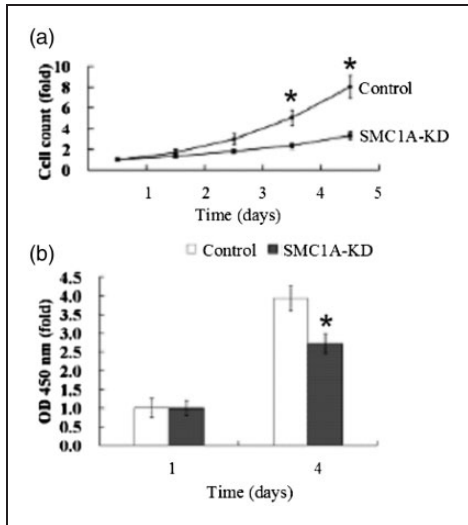


Figure 2. Structural maintenance of chromosomes 1A small interfering RNA inhibited the proliferation of HT-29 cells. (a) Cell number was analysed using an ArrayScanTM XTI High Content System reader in HT-29 cells infected with control or SMC1A siRNA (Control or SMC1A-KD cells, respectively). (b) Cell proliferation was analysed using a 5-bromo-2-deoxyuridine incorporation assay. Data presented as mean \pm SD ($n = 3$).

* $P < 0.05$, Student's *t*-test.

knockdown. These present data suggest that the SMC1A protein may enhance the tolerance to DNA damage, promote cell-cycle progression and inhibit apoptosis, leading to increased chemoresistance.

The present study had a number of limitations. First, only siRNA was employed to knockdown *SMC1A* gene expression in one human colorectal cancer cell line. It will be necessary to examine the effects of *SMC1A* gene knockdown in other colorectal cancer cells. Secondly, this present study focused on the role of SMC1A in the proliferation and apoptosis of colorectal cancer cells. Additional experiments need to be undertaken, such as investigating the role of cell cycle- and apoptosis-related proteins, in order to be able to understand the underlying mechanisms by which SMC1A regulates chemosensitivity in colorectal cancer cells.

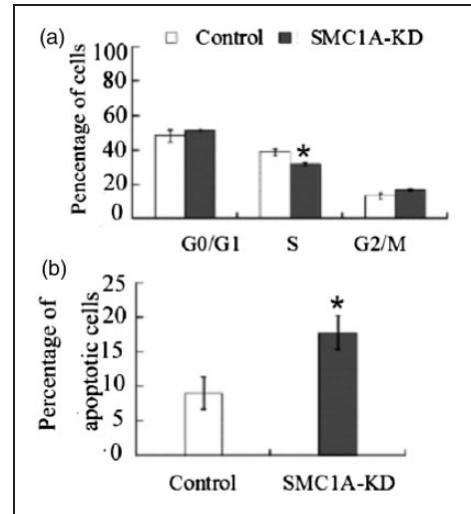


Figure 3. Structural maintenance of chromosomes (SMC) 1A small interfering RNA (siRNA) inhibited cell-cycle progression and induced apoptosis of HT-29 cells. (a) Flow cytometric analysis of the proportion of cells in different cell-cycle phases of HT-29 cells infected with control or SMC1A siRNA (Control or SMC1A-KD cells, respectively). (b) Flow cytometric analysis of the proportion of apoptotic cells in Control or SMC1A-KD cells. Data presented as mean \pm SD ($n = 3$).

* $P < 0.05$, Student's *t*-test.

In conclusion, by employing a loss-of-function approach to knockdown *SMC1A* gene expression, this present study has demonstrated that loss of SMC1A mRNA and protein lead to reduced cell proliferation and increased apoptosis and chemosensitivity to oxaliplatin in a human colorectal cancer cell line. To our knowledge, these results provide the first evidence that SMC1A plays an oncogenic role in colorectal cancer. These current findings also suggest that SMC1A might be a promising target for colorectal cancer therapy in future.

Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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