Structural Maintenance of Chromosomes 4 is a Predictor of Survival and a Novel Therapeutic Target in Colorectal Cancer

Xiao-Dong Feng¹, Qi Song¹, Chuan-Wei Li¹, Jian Chen², Hua-Mei Tang³, Zhi-Hai Peng², Xue-Chun Wang¹*

Abstract

Background: Structural maintenance of chromosomes 4 (SMC-4) is a chromosomal ATPase which plays an important role in regulate chromosome assembly and segregation. However, the role of SMC-4 in the incidence of malignancies, especially colorectal cancer is still poorly understood. Materials and Methods: We here used quantitative PCR and Western blot analysis to examine SMC-4 mRNA and protein levels in primary colorectal cancer and paired normal colonic mucosa. SMC-4 clinicopathological significance was assessed by immunohistochemical staining in a tissue microarray (TMA) in which 118 cases of primary colorectal cancer were paired with noncancerous tissue. The biological function of SMC-4 knockdown was measured by CCK8 and plate colony formation assays. Fluorescence detection has been used to detect cell cycling and apoptosis. Results: SMC-4 expression was significantly higher in colorectal cancer and associated with T stage, N stage, AJCC stage and differentiation. Knockdown of SMC-4 expression significantly suppressed the proliferation of cancer cells and degraded its malignant degree. Conclusions: Our clinical and experimental data suggest that SMC-4 may contribute to the progression of colorectal carcinogenesis. Our study provides a new therapeutic target for colorectal cancer treatment.

Keywords: Structural maintenance of chromosomes 4 - colorectal cancer - potential therapeutic target

Introduction

Colorectal cancer (CRC) is one of the most frequent malignances in the world. It is the third most common cancer and the third leading cause of cancer death in the United State (Siegel et al., 2014). Although dramatic reduction in cancer mortality and prolong patient survival with surgical resection and chemotherapy has been achieved clinically (Yan et al., 2010). However, tumor recurrence and the chemoresistance is the main factor for the failure of therapy (Chang, 2011). CRC arises through the progressive accumulation of mutations in oncogenes and tumor-suppressor genes (Samsom et al., 2007; Petrova et al., 2008). Despite several genes have been proving that relate to genesis of CRC, the exact mechanisms underlying he development and progression CRC are still poorly understood (Goel and Boland, 2010). Throughout the articles relate to CRC in the journal ACJCP over the past two years, it is not difficult to find that the novel molecular markers with potential of treatment and prognosis have captured more and more attention (Chen et al., 2013; Zhu et al., 2013). Given this consideration, it is imperative to seek out a key gene associated with the progression and pathogenesis.

Structural maintenance of chromosomes (SMC) proteins is a member of the chromosomal ATPases family. They are highly conserved from bacteria to human beings, and play critical roles in regulations of higher-order chromosome organization and dynamics (Losada and Hirano, 2005). Eukaryotes have three SMC complexes which consisting of heterodimeric pairs of six different SMC proteins (Griese et al., 2010). SMC-4 also known as chromosome-associated polypeptide C (CAP-C) or XCAP-C homolog, it is a protein that in humans is encoded by the SMC-4 gene which is located on chromosome 3q26.1 (Nishiwaki et al., 1999). Recent studies have indicated SMC-4 is up-regulated and play an important role in many malignancies, including primary liver cancer (Zhou et al., 2012), epithelial ovarian cancer (Lu et al., 2004), glioma (Takahashi et al., 2012), breast (Kulawiec et al., 2008) and lung cancers (Bidkhori et al., 2013). However, its expression and potential role in the pathogenesis in CRC remain unknown.

In this study, we first examined the expression of SMC-4 in colorectal cancer and paired normal tissues using qRT-PCR, Western blot and immunohistochemistry, and then we tested the influence of SMC-4 expression on RKO cell growth, migration and invasion. The results suggested that SMC-4 might serve as a significant independent prognostic factor and potential target of CRC drug therapy.

¹Department of Basic Medicine, Taishan Medical University, Taian, ²Department of General Surgery, ³Department of Pathology, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, China *For correspondence: xuechun_wang@163.com
Human Tissue Specimens and Patients Information

A total 118 human colon tumor specimens used in this study obtained from the Colorectal Cancer Tissue Bank at Shanghai Jiaotong University Affiliated First People’ Hospital between January 2001 and December 2003. The specimens came from colon cancer patients who undergone tumor resection, and none of them received either chemotherapy or radiotherapy before surgery. The diagnosis was confirmed by 2 pathologists at least. Pathological staging was based on the standard of American Joint Committee on Cancer (AJCC).

Our population comprised 67 male and 51 female patients who permitted operation by the same surgical team with the General Surgery Department of Shanghai Jiaotong University Affiliated First People’ Hospital. The median age and median patient follow-up time was 67.5 years (25-84 years) and 67.5 months after surgery (18-80 months). All of the tissue specimens were obtained with the patients’ consent, and the study was approved by the Institutional Review Boards of Shanghai Jiaotong University Affiliated First People’ Hospital Medical Center.

RNA extraction and quantitative real-time PCR

All of 38 primary tumor and paired normal mucosa of colon specimens stored at -80°C were used for qPCR analysis. Total RNA was extracted according to the manufacturer’s instruction (TRIzol, Invitrogen, USA). First-strand cDNA was synthesized using the RNA PCR Kit (Takara, Japan) according to the operator’s manual. SMC-4 gene was amplified using forward primer 5’-GAGAAAAATTCGAGACTTTT-3’ and antisense primer 5’-TCTGAAATGTCTTGTGTCCTA-3’, and glyceraldehyde3-phosphate dehydrogenase (GAPDH), sense 5’-TGACTTCAACAGCGACACCA-3’, and antisense 5’-CACCCTGTCTGTAGCCAAA-3’, was used as an internal control. qPCR was performed on a Mastercycler ep Realplex (Eppendorf) using the IQTM SYBR Green Supermix Kit (BIO-RAD) according to the manufacturer’s protocol. Cycling conditions were configured as follows: initial denaturation (10min at 95°C) and then 40 cycles of denaturation (10 sec at 95°C), annealing (15 sec at 58°C), extension (1 min at 72°C), and with a final extension at 72°C for 5 min. The fold change \((2^{-\Delta\Delta C_t}}\) of SMC-4 expression was calculated using the formulas: SMC-4\(\Delta C_t = \)Avg.SMC-4\_Ct-Avg. GAPDH\_Ct, and SMC-4\(\Delta\Delta C_t = SMC-4\Delta C_t\_tumor-SMC-4\Delta C_t\_normal\).

Western blot analysis

Four patients’ paired tissue protein was extracted by using the Radio Immunoprecipitation Assay (RIPA) lysis buffer (50mM Tris pH7.4, 150 mM NaCl, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and the concentration was determined using the BCA protein assay kit (Beyotime Biotechnology, jiangsu, China). Equivalent paired protein were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. And then the membranes were blocked in 5% fat free milk with 0.1% Tween 20 for 1 hour at room temperature. Followed by incubation with primary antibodies (1:1000 dilute for SMC-4, 1:1000 dilution for GAPDH, all purchased from Abcam, UK). Then blots were incubated with goat-anti-mouse immunoglobulin horseradish peroxidase conjugate secondary antibodies and detected by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA) and autoradiography.

Tissue microarray (TMA) construction and Immunohistochemistry

Formalin-fixed, paraffin-embedded samples comprises of tumor and paired normal were taken back from the Department of Pathology of Shanghai Jiaotong University Affiliated First People’ Hospital. Representative areas of the tissue on TMA were identified by HE-stained slides, and 2mm diameter cores were punched out from the paraffin blocks with a punch instrument. HE-staining was performed on TMA for confirmation of tumor and normal tissue. All samples were evaluated by 2 pathologists independently who were blinded to patients’ information.

Immunohistochemical analysis was performed on the TMA. Immunostaining was performed using the primary antibody against SMC-4 (1:200; Abcam, UK), and incubated with the secondary antibody (Genetech, Shanghai, China). Mayer’s hematoxylin was used to counterstain finally.

The evaluation was based on the staining intensity of SMC-4: 0 (negative); 1 (weak); 2 (strong). The cases were re-examined by another pathologist in the event of a discrepancy in scoring.

Cell Culture and construction of the stable SMC-4 expressing cell line

The human colon cancer cell line RKO, HT29, SW620, LoVo and HCT116 (purchased from Center of Shanghai Institutes for Biological Sciences, Type Culture Collection of Chinese Academy of Sciences) was cultured in DMEM medium supplemented with 10%FBS (Gibco) and 1% streptomycin, penicillin. All the cell lines were in a 5% humidified atmosphere at 37°C.

To determine the biofunction of SMC-4, RKO cell line were transfected with shRNA of SMC-4 and GFP-tag by Lipofectamine 2000 according to the manufacturer’s instructions. At 48 h post-transfection of the synthesized shRNA, the cells were used for identification and research.

Cell proliferation assay and colony formation assay

For cell proliferation assay, each cell line was seeded in the 96-well plates at a density of 2000 cells per well and cultured for 24, 48, 72, 96 and 120 h, respectively. Then removed the supernatant and 100 μL DMEM culture medium containing 10 μL CCK8 (Dojindo, Shanghai, China) was added into each well for another 2 h at 37°C. Results were read on a microplate luminometer at the absorbance of 450 nm.

For plate colony formation assay, cells were collection and plated in a 6-well plate at the density of 800 cells per well and incubated at 37°C for 14 days. After washed two times with PBS, fixed in paraformaldehyde for 30 min, cells were stained with GIMSA solution for 20 min and images were obtained.
According to the manufacturer’s instructions, the cells were harvested, rinsed twice with PBS and resuspended in 1×binding buffer to each tube, and analyzed by flow cytometry immediately.

**Statistical analysis**

Data were shown as mean±SD. The Student’s t-test was used to determine the statistical significance of differences between the two groups. The chi-square or Fisher’s exact tests were used to calculate the relationships between SMC-4 expression in different tissues and clinicopathological parameters. The overall survival (OS) rates and disease-free survival (DFS) were calculated using the Kaplan-Meier method, and the differences were examined by the log-rank test. \( p<0.05 \) was considered statistically significant. All statistical analyses performed by using the SPSS 19.0 (SPSS Inc, Chicago, IL).

**Results**

**SMC-4 was up-regulated in colorectal cancerous tissue**

We first examined the SMC-4 mRNA expression in 38 randomly selected, paired specimens by qRT-PCR. Among these, 22 (57.9%) colorectal cancerous tissues increased by greater than 2-fold compared with the non-cancerous (Figure 1A). The SMC-4 mean relative quantification in cancerous tissues were much higher than the protein expression in 4 samples which selected from normal tissues (Figure 1B).

**Relationship between SMC-4 TMA Immunohistochemistry and clinicopathological parameters**

To further evaluated relationship between SMC-4 expression and clinicopathologic features of colorectal cancer, immunohistochemistry was performed to detect the expression of SMC-4 on the tissue microarray containing 118 paired specimens. In interphase cells, the majority of the condensin complex is found in the cytoplasm so we found positive SMC-4 staining was predominantly localized in the cytoplasm of colonic epithelial and tumor cells (Figure 2). And of the 118 paired specimens TMA, 9 (7.6%) normal specimens showed strong positive SMC-4 expression, 26 (22%) specimens showed weak staining, and 83 (70.4%) specimens were negative staining. In contrast, SMC-4 expression was

**Table 1. Expression of SMC 4 in Normal Colorectal Mucosa and Cancerous Tissues**

| Tissue sample | n  | Expression of SMC 4 | \( p \) value |
|---------------|----|--------------------|--------------|
| Normal mucosa | 118| Negative (74, 68)   |              |
|               |    | Weak (22, 19)       |              |
|               |    | Strong positive (2, 4) | \( <0.001^* \) |
| Tumor         | 118| Negative (72, 54)   |              |
|               |    | Weak (8, 8)         |              |
|               |    | Strong positive (2, 3) | <0.01       |

* Significant difference; \( P \)-value is based on chi-square test

PI. Gently vortex the cells and incubated for 15 min at room temperature (25°C) in the dark. Added 400 μL of 1×binding buffer to each tube, and analyzed by flow cytometry immediately.

**Cell cycle analysis**

Cell cycle analysis was performed following PI staining (Rainbio, Shanghai, China). Each cell was seeded into three 6 cm plate and incubated at 37°C, 5% CO\(_2\), then collected by trypsinization, centrifuged at 1000 g for 5 min, washed twice in cold PBS, fixed in 70% ethanol at 4°C overnight. Wash cells in cold PBS again, then treated with 20 μl RNase A (20 μg/ml) in 500 μl PBS at 37°C for 30 min. Next cell nuclei were stained with 400 μl PI (50 μg/ml) at 4°C for 30 min. The cells (5×10\(^5\)) were analyzed for their DNA content using flow cytometry.

**Apoptosis and cell cycle analysis**

We first examined the SMC-4 mRNA expression in 38 paired colorectal cancerous tissue specimens compared with in normal mucosa specimens. 2\(^\Delta\Delta\text{Ct}\) as a logarithmic scale was used to represent the fold change of in quantitative rt-PCR (B) SMC-4 protein expression in 4 paired colorectal tissues measured by Western blot analysis.

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**Figure 1. Expression of SMC-4 in Colorectal Tumor Tissues and Adjacent Normal Mucosa.** A) Relative expression of SMC-4 in 38 matched colorectal cancerous tissue specimens compared with in normal mucosa specimens. 2\(^\Delta\Delta\text{Ct}\) as a logarithmic scale was used to represent the fold change of in quantitative rt-PCR (B) SMC-4 protein expression in 4 paired colorectal tissues measured by Western blot analysis.
much higher in tumor tissues with strong positive staining in 56 (47.6%) specimens, weak positive staining in 43 (36.3%) specimens, negative staining in only 19 (16.1%) specimen (Table 1). Moreover, 43 (76.8%) samples of SMC-4 strong positive expression showed T3-T4 tumor invasion among total 56 samples, which suggested that SMC-4 was closely related to tumor growth of colorectal cancer (Table 2).

Associations between clinicopathological parameters and SMC-4 expression are summarized in Table2. According to the statistical analysis, up-regulated SMC-4 was associated with T stage ($p<0.001$), N stage ($p=0.02$), AJCC stage ($p=0.011$) and differentiation ($p=0.008$), but no correlations were found with age, gender, tumor location, M stage, and vessel invasion (Table 2).

High SMC-4 expression associated with poor clinical outcome in human colorectal cancer

To assess the relationship between tumor SMC-4 expression and disease metastasis and patient survival, we used Kaplan-Meier curves and log rank test to show that the expression of SMC-4 was significantly associated with overall survival (OS) and disease-free survival (DFS) rate ($p<0.001$, Figure 3A, B). Patients with strong positive SMC-4 expression had an obviously lower OS and DFS rate than patients with negative SMC-4 expression. Moreover, the estimated mean OS was markedly different between patients with SMC-4 positive and negative tumors (74.33±4.15 and 52.96±16.03 months, respectively; $p=0.001$). The estimated mean DFS time was 73.47±5.89 and 40.46±19.44 months for subjects with SMC-4 positive and negative tumors ($p<0.001$).

Increased SMC-4 expression inhibits the proliferation of colorectal cancer cells

The cell line suitable for SMC-4 knockdown transfection was selected from RKO, HT29, SW620, LoVo and HCT116. The results of western blot show that RKO has the highest SMC-4 protein expression among 5 cell lines and HCT116 was the lowest (Figure 4A). So we selected RKO as the target in the following study. We used Western blotting to show that SMC-4 specific shRNA
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significantly reduced the SMC-4 protein level while mock-transfected and control shRNA groups showed no difference in SMC-4 levels (Figure 4B).

Since SMC-4 was up-regulated in colorectal cancerous tissue and linked to the poor survival of colorectal cancer patients. We conducted knockdown of SMC-4 expression to evaluate the effect on cell proliferation. In the CCK8 assay, the results suggested that an obvious inhibition cell grow in the group of SMC-4 knockdown compared with the mock and control group at 24, 48, 72, 96 and 120 h time points (Figure 4C). Consistent with the CCK8 assay, plate colony formation assay also proved that SMC-4 knockdown caused 71.4% inhibition of cancer cell clonogenicity compared with mock and control groups approximately (Figure 4D).

Knockdown of SMC-4 attenuated the migration and invasion of colorectal cancer cells

Because SMC-4 expression was correlated with distant metastasis of colorectal cancer, we here investigated the role of SMC-4 in migration and invasion. We used migration chambers to appraise the effect of SMC-4 silencing on migration and invasion potency of RKO cells. The SMC-4 knockdown group showed a significant lower...
migratory ability than those of control cells (Figure 5A, C). The similar phenomenon was observed in invasion assay (Figure 5B, D). These results suggest a functional role for SMC-4 in colorectal cancer metastasis.

**Knockdown of SMC-4 prolonged G2/M phase of RKO cells and made them apoptosis**

Because SMC-4 depletion led to down-regulation of proliferation of colorectal cells, we next investigated how SMC-4 might affect them. Flow cytometric analysis PI labeling demonstrated that depletion of SMC-4 causes a more pronounced G2/M arrest, but not in the mock and control groups (Figure 6A). We next analyzed the incidence of apoptosis in the three different groups also by flow cytometry analysis and showed higher numbers of apoptotic cells in SMC-4 knockdown cells than in mock and control group (Figure 6B).

**Discussion**

Colorectal cancer is the common malignant tumor worldwide with a low survival rate and the treatment is difficult. SMC-4 is an indispensable factor involved in chromosome condensation which has been implicated in several malignancies (Lu et al., 2004; Kulawiec et al., 2008; Takahashi et al., 2012; Zhou et al., 2012; Bidkhori et al., 2013). Abnormal SMC-4 expression in tumor tissues suggests that SMC-4 is significant in cancer pathogenesis and progression. However, the expression of SMC-4 and its potential role in colorectal cancer remain elusive. To the best of our knowledge, this is the first report on the expression and function of SMC-4 in colorectal cancer.

In the present study, we first show that the mRNA and protein expression levels of SMC-4 in colorectal cancer were significantly higher than in the compared normal tissue. It was suggested that SMC-4 was elevated at both transcriptional and posttranscriptional levels. In addition, we found a significant association between SMC-4 expression with disadvantageous clinicopathologic factors, including T stage, N stage, AJCC stage and differentiation. Furthermore, patients with strong positive SMC-4 expression had an obviously lower OS and DFS rate than patients with negative SMC-4 expression. Jinushi et al demonstrated that higher miR-124-5p expression correlated with a higher OS of CRC by inhibited the expression of SMC-4 which confirmed our results indirectly (Jinushi et al., 2014). We also investigated the biological effects on RKO cells imposed by knockdown SMC-4. The results also indicated that knockdown of SMC-4 plays a suppressive role in the proliferation, cell cycle and apoptosis of colorectal cancer cells.

The mechanism by which SMC-4 contributes to tumorigenesis and tumor progression has not been well illuminated. The human SMC-4 gene is located on chromosome 3q26.1. The chromosome damage of this region related to multiple tumorigenesis (Hui et al., 2005; Thean et al., 2010; Kawachi et al., 2013). SMC-4 is a core subunit of condensin I and II, large protein complexes involved in chromosome condensation (Losada and Hirano, 2005). A possible explanation for tumorigenesis of SMC-4 is that it acts as a part of the condensin complex, and functions in the condensation of chromosomes during mitosis (Harvey et al., 2002). Besides, SMC-4 has been reported to play a pivotal role in DNA replication and recombination in breast cancer (Wang et al., 2005). Therefore, altered SMC-4 expression might affect chromosomal stability, and increase dsDNA breaks and unique chromosomal rearrangements (Kulawiec et al., 2008). Hagstrom et al used RNA interference technology have demonstrated that chromosomes depleted of SMC-4 have altered morphology and fail to segregate (Hagstrom et al., 2002). Similarly, Zhai et al. found that the knockdown of SMC-4 led to a chromosomal separation deficiency (Zhai et al., 2011). These results are similar to ours that SMC-4 knockdown causes a more pronounced G2/M arrest in RKO cells. Moreover, Ono et al revealed depletion of condensing I and II specific subunits causes a distinct defect in chromosome morphology (Ono et al., 2003). In the p53 network, some research also identified SMC-4 whose changes in expression suggest that SMC-4 may affect chromosomal stability through p53 pathway (Wirtenberger et al., 2006). Although the knockdown experiments alone is insufficient to provide strong evidence. However, our data prove that SMC-4 has great potential value of prognosis and treatment for colorectal cancer. Our future work will increase in vivo study and aim to dissect the underlying molecular mechanism responsible for alterations in SMC-4 expression in CRC. Ultimately, we hope to find a new effective therapeutic strategy.

In conclusion, we found that SMC-4 expression in CRC tissues was significantly upregulated compared with its adjacent normal tissues and SMC-4 knockdown promoted carcinogenesis, migration and invasion. These suggest that SMC-4 plays an important role in incidence and development of CRC. We provide the basis for prognosis and new treatment option of colorectal cancer. The preliminary results above need to be confirmed in a larger, prospective, controlled, clinical study.

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