SIRT1 promotes epithelial–mesenchymal transition and metastasis in colorectal cancer by regulating Fra-1 expression

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ABSTRACT

Understanding molecular mechanisms of colorectal cancer (CRC) metastasis is urgently required for targeted therapy and prognosis of metastatic CRC. In this study, we explored potential effects of silent mating type information regulation 2 homolog 1 (SIRT1) on CRC metastasis. Our data showed that ectopic expression of SIRT1 markedly increased the migration and invasion of CRC cells. In contrast, silencing SIRT1 repressed this behavior in aggressive CRC cells. Tumor xenograft experiments revealed that knockdown of SIRT1 impaired CRC metastasis in vivo. Silencing SIRT1 in CRC cells induced mesenchymal–epithelial transition (MET), which is the reverse process of epithelial–mesenchymal transition (EMT) and characterized by a gain of epithelial and loss of mesenchymal markers. We provided a mechanistic insight toward regulation of Fra-1 by SIRT1 and demonstrated a direct link between the SIRT1-Fra-1 axis and EMT. Moreover, SIRT1 expression correlated positively with Fra-1 expression, metastasis and overall survival in patients with CRC. Taken together, our data provide a novel mechanistic role of SIRT1 in CRC metastasis, suggesting that SIRT1 may serve as a potential therapeutic target for metastatic CRC.

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Introduction

Colorectal cancer (CRC) is the third most common malignancy and the fourth leading cause of cancer death worldwide [1]. Despite the achievements that have been made to date, the radical cure of CRC still remains a challenging task. It is now generally accepted that metastasis is the main cause of death in patients with CRC due to resulting in tumor resistance to conventional therapies and a poor overall survival [2,3]. In recent years, genetic alterations in CRC have been extensively studied [4–6]. However, the relevant factors that contribute to metastasis are still not well determined, which is urgently required for the targeted therapy and prognosis of metastatic CRC.

Silent mating type information regulation 2 homolog 1 (SIRT1) is a NAD+–dependent deacetylase involved in many biological processes, including cellular metabolism, stress response, and aging [7–9]. Over the past few decades, SIRT1 has more been implicated in the initiation and progression of various malignancies [10,11]. Previous studies have reported that SIRT1 is upregulated in leukemia, glioblastoma, prostatic, lung, breast, liver and colorectal cancer [11–17]. Changes in SIRT1 potentially exert pleiotropic effects that alter cellular functions including proliferation, apoptosis and metastasis [18–21]. Additionally, SIRT1 overexpression is associated with poor prognosis in CRC and other malignancies such as lung cancer [14,16], which further supports its potential role in the aggressive behavior of these cancers.

Despite above findings, the role and molecular mechanisms of SIRT1 on CRC metastasis are less understood. In this study, we found that ectopic expression of SIRT1 drove migration and invasion in human CRC cells. Knockdown of SIRT1 repressed migration and invasion in vitro and metastasis in vivo of CRC cells. Importantly, these functional effects of SIRT1 were exerted through regulating epithelial–mesenchymal transition (EMT) in a Fra-1–dependent manner. Moreover, SIRT1 expression correlated positively with Fra-1 expression, metastasis and overall survival in patients with CRC. Thus, our findings provide a novel mechanistic role of SIRT1 in CRC metastasis and a rationale for clinically exploring the use of SIRT1 inhibitors in the therapy of metastatic CRC.

Materials and methods

Cell lines and reagents

LoVo, HCT116, and HT29 cell lines were obtained from Shanghai Cell Collection (Shanghai, China). SW480 and SW620 cells were from the American Type Culture
Collection (ATCC, Manassas, VA, USA). All cells were cultured in DMEM medium with 10% FBS (Gibco) at 37 °C in a humidified atmosphere containing 5% CO2. For the generation of stable cell lines, SW480 cells were transfected with empty vector (pcDNA3.1) or plasmid-expressing SIRT1. Viafect™ Transfection Reagent was purchased from Promega (Madison, WI, USA). The siRNAs targeting Fra-1 and tenovin-6 were from Santa Cruz.

**Tumor xenograft in NOD/SCID mice**

All mice used in these experiments were maintained at the institutional facilities corresponding to the Guide for the Care and Use of Laboratory Animals. All experimental procedures involving animals were performed in accordance with the institutional ethical guidelines from the Animal Ethics Committee of the Third Military Medical University. Cells were injected into the spleen of 6-week-old NOD/SCID mice at 1 × 10^7 cells/injection site. The mice were sacrificed after 6 weeks, and liver metastases were enumerated. The tumor size was measured using a caliper. The tumor volume was calculated as follows: (length × width)^2/2. The experimental procedures were approved by the Ethics Committee of the Second Military Medical University.

**Immunohistochemical staining**

The tumors dissected from mice were fixed in 4% paraformaldehyde and subsequently embedded in paraffin wax. Tissue microarrays (HCM-Asc180S04-U4) containing 90 CRC and 90 paired non-CRC counterparts were provided by Outdo Biotech Co., Ltd. (Shanghai, China). Immunohistochemical (IHC) staining was performed using the streptavidin–biotin–peroxidase complex method. Antigen retrieval was conducted by heating the tissues with the antigen retrieval solution (Dako, Carpinteria, CA, USA). Then, the tissues were incubated with primary antibodies. Antibodies used in the study were listed in Supplementary Table S1. Scoring for IHC staining was carried out using a semi-quantitative method based on the staining intensity and the area of positive cells. Briefly, the staining intensity was divided into 4 grades: negative, 0; weak, 1; moderate, 2; and strong, 3. The area of positive cells was divided into 5 grades: 0: 0% ; 1: 1–25%; 2: 26–50%; 3: 51–75%; and 4: 76–100%. The final staining scores were calculated by multiplying the above two scores, resulting in an overall score that ranges from 0 to 12. SIRT1 and Fra-1 expressions were defined as the following rule: 0–4 (low); 5–12 (high). The staining results were assessed and confirmed by two independent pathologists blinded to the clinical data. The clinicopathological information of all cases was made publicly available at the company website (http://www.superchip.com.cn/).

**Transwell and wound healing assays**

Transwell assay was performed in 24-well plates. Cells that were seeded in medium without serum were plated in the upper chamber, and medium containing 20% FBS was added to the lower chamber as a chemoattractant. After 48 h of incubation at 37 °C, the cells on the upper surface of the filter were removed carefully with a cotton swab. The cells that migrated/invaded to the underside of the membrane were fixed in methanol and stained with 0.5% crystal violet, and enumerated under a microscope. The values were calculated by averaging the total number of cells from three filters. For wound healing assays, cells were seeded on six-well plates and grown to confluency. Then, the cells were gently scratched with tips to create a mechanical wound. Images were taken at 0 and 72 h using a microscope.

**Quantitative real-time PCR analysis**

Total RNA was extracted from the cells using RNAiso (Takara) according to the manufacturer’s protocol. For quantitative real-time PCR (qRT-PCR), reverse transcription was conducted as the protocol of PrimeScript™ RT reagent kit (Takara); qRT-PCR was performed with SYBR premix Ex Taq (Takara) on CXF96 Real Time PCR Detection System (Bio-Rad, USA). Actin mRNA was used to normalize mRNA expression for equal loading, and the results were representative of at least three independent experiments. The sequences of the primers were shown in Supplementary Table S2.

**Immunofluorescence analysis**

Cells were grown on glass cover-slips in a 24-well plate and washed three times with PBS before being fixed in 4% paraformaldehyde, and permeabilized for 20 min with 0.2% Triton X-100 at room temperature (RT). Cells were blocked with 10% FBS (Gibco) in PBS for 1 h at RT, and then probed with primary antibodies. Primary antibodies were shown at Supplementary Table S2. Secondary antibodies were donkey anti-rabbit IgG-Alexa Fluor 647, or donkey anti-mouse IgG-Alexa Fluor 647 (Invitrogen). Cells were further washed in PBS and the nuclei were stained with DAPI (Sigma) for 6 min. The slides were mounted and visualized using a fluorescence microscope.

**Western blot analysis**

Cells were harvested and lysed in the lysis buffer for 30 min at 4 °C. Total cell extracts were separated in 10% SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred on NC membrane. The membrane were then blocked in 5% milk for 2 h at RT and incubated with antibody overnight at 4 °C. After washing with phosphate buffered saline with Tween-20 (PBST) and incubating with secondary antibody at a dilution of 1:3000 in PBST, immunocomplexes were visualized by using SuperSignal West Femto Chemiluminescent Substrate (Pierce). Antibodies used in the study were listed in Supplementary Table S2.

**Luciferase reporter assay**

The luciferase reporter assay was performed by transfecting the reporter construct into the indicated cell lines. The pRL-SV40 vector was co-transfected in each experiment as an internal control for transfection efficiency. At 36 h post-transfection, the luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s protocol. All experiments were carried out in triplicate.

**Statistical analysis**

Unpaired/paired, two-sided t test was used to calculate the statistical significance of the experimental data. The Kaplan–Meier survival curves were used for overall survival analysis. The level of significance was set as *p < 0.05 and **p < 0.01. All data were presented as the means ± standard deviation (SD). Statistical analysis and graphs were generated using the GraphPad Prism software (GraphPad Software, San Diego, CA) and Microsoft Excel.

**Results**

**SIRT1 enhances colorectal cancer cell migration and invasion**

To study the role of SIRT1 in tumor metastasis, we utilized five distinct cell lines that differ in their migratory/metastatic abilities. Among these cells, LoVo, SW620, and HCT116 have been known to possess highly migratory/metastatic abilities (Fig. 1A and Supplementary Fig. S1A), while HT29 and SW480 are not [22–24]. Consistent with previous report [16], we found that there was a higher expression of SIRT1 in high-metastatic versus low-metastatic tumor cells, whether mRNA or protein level (Fig. 1B and Supplementary Fig. S1B), and SIRT1 expression was significantly associated with migratory abilities of tumor cells (Fig. 1C). Then, we established SW480 cell lines that stably express SIRT1 (designated as SW480-SIRT1, Supplementary Fig. S1C), and analyzed their migratory and invasive capacities in vitro. Compared to the control, SIRT1 overexpression markedly enhanced the number of migratory and invasive cells (Fig. 1D and E, Supplementary Fig. S1D and E). To further examine whether endogenously high SIRT1 contributes to migration and invasion, we blocked SIRT1 in SW620 and LoVo cells (designated as SW620-shSIRT1 and LoVo-shSIRT1) via a lentivirus-based approach. The knockdown efficiency of SIRT1 was checked via western blotting analysis (Supplementary Fig. S1F). Knockdown of SIRT1 significantly impaired their migratory and invasive capacities compared to the control (Fig. 1F, G and H, Supplementary Fig. S1G and H). Taken together, our findings show SIRT1 enhances colorectal cancer cell migration and invasion in vitro.

**SIRT1 is essential for tumor metastasis in mice**

In order to investigate the function of SIRT1 on tumor metastasis in vivo, SW620-shSIRT1, LoVo-shSIRT1 and their corresponding control cells were injected into the spleen of each mouse. Six weeks later, mice were sacrificed and necropsied to determine the tumor growth and metastatic pattern. Our data indicated, irrespective of silencing SIRT1 or not, established tumors in situ showed basically the same mean weight and volume (Fig. 2A and B). The knockdown efficiency of SIRT1 was further confirmed in established tumors by Immunohistochemistry analysis (Fig. 2C). Importantly, all of the mice that received injections of the control
cells developed micrometastases in liver, which were hardly seen in animals injected with SW620-shSIRT1 cells (Fig. 2D and E). Moreover, the number of liver metastatic foci decreased dramatically in the SW620-shSIRT1 group when compared with that in the control group (Fig. 2F). The same effect was observed in LoVo-shSIRT1 and corresponding control cells (Supplementary Fig. S2A and B). These results suggested that SIRT1 promoted the metastatic process of CRC cells in vivo.
SIRT1 promotes metastasis by regulating epithelial–mesenchymal transition

Growing evidence indicates that the EMT is a major event involved in tumor metastasis [25–27]. A widely accepted marker for EMT is loss of epithelial markers (E-cadherin and occluding) with concomitant upregulation of mesenchymal markers (vimentin and fibronectin) [28–30]. To understand whether SIRT1 promotes CRC metastasis by regulating EMT, we performed a qRT-PCR analysis of the expression of E-cadherin, occluding, vimentin and fibronectin in SW620-shSIRT1 cells. Our data showed that knockdown of SIRT1 led to MET with upregulation of E-cadherin and occluding, and downregulation of vimentin and fibronectin (Fig. 3A). The expression changes of EMT markers were confirmed at the protein level (Fig. 3B and C). The same effect was also observed in LoVo-shSIRT1 cells (Fig. 3C). Moreover, the de novo expression of SIRT1 reverted the MET property of SW620-shSIRT1 cells (Fig. 3D).

To further verify whether SIRT1 contributes to EMT, we blocked SIRT1 in SW620 cells with tenovin-6 that specifically inhibits SIRT1 deacetylase activity. We measured the effect of tenovin-6 on the cell viability of CRC cells. The results demonstrated that tenovin-6 (1 μmol/L) did not impair the cell viability (Supplementary Fig. S3A). Consistent with knockdown of SIRT1, a clear effect on EMT was observed in the presence of tenovin-6 (1 μmol/L) for 48 h in SW620 cells (Fig. 3E). Taken together, these results supported the finding that SIRT1 promotes EMT-mediated CRC cell metastasis.

SIRT1 regulates Fra-1 in colorectal cancer cells

Our previous study and many others have demonstrated that the AP-1 transcription factor Fra-1 is involved in tumor-associated EMT [24,31,32]. Moreover, Fra-1 has been proposed as a gatekeeper of the EMT program during CRC metastasis [33]. Intriguingly, we detected a clear correlation between SIRT1 and Fra-1 levels in CRC cell lines (Fig. 4A). These findings prompt us to investigate whether SIRT1 regulates Fra-1. Firstly, qRT-PCR was performed to examine the mRNA expression of Fra-1 in SW620-shSIRT1 and the control cells. Our data showed knockdown of SIRT1 downregulated Fra-1 expression at mRNA level (Fig. 4B). A reduced Fra-1 promoter activity was also
Fig. 3. SIRT1 promotes metastasis by regulating epithelial–mesenchymal transition. (A) qRT-PCR was used to detect changes in the mRNA expression of EMT-associated genes in SW620-shSIRT1 and the control cells. Values are means ± SD. *p < 0.05, **p < 0.01. (B) Immunofluorescence staining was performed to detect changes in the protein expression of EMT-associated genes in SW620-shSIRT1 and the control cells. Nuclei were counterstained with DAPI (×100). (C) Western blot analysis was performed to detect changes in the protein expression of EMT-associated genes in SW620-shSIRT1 and LoVo-shSIRT1 and their control cells. (D) Western blot analysis was performed to detect changes in the protein expression of EMT-associated genes in SW620-shSIRT1 cells after the de novo expression of SIRT1. (E) Western blot analysis was used to detect changes in the protein expression of EMT-associated genes in SW620 cells treated with tenovin-6.
evident from luciferase reporter assays (Fig. 4C). The immunofluorescence and western blotting analysis further supported the finding that SIRT1 regulates Fra-1 in CRC cells (Fig. 4D and E).

**SIRT1 regulates EMT in a Fra-1-dependent manner**

Based on the above findings, we further investigated whether SIRT1 regulated EMT in a Fra-1-dependent manner. As shown in Fig. 5A, knockdown of SIRT1 yielded a substantial decrease in Fra-1 compared to control cells, which was associated with downregulation of vimentin and upregulation of E-cadherin. The same effect was observed in tenovin-6 treated SW620 cells (Fig. 5B). Next, we confirmed the effect of Fra-1 on EMT markers in SW620 cells. Western blotting analysis indicated that silencing Fra-1 after transfecting with siRNA led to decreased vimentin and increased E-cadherin protein levels (Fig. 5C). Moreover, we found that the appearance of SW480 cells with adherent polygonal phenotype was replaced by a scattered spindle-shaped invasive phenotype under SIRT1 expression. However, the mesenchymal-like morphological conversion driven by SIRT1 was substantially reverted after knockdown of Fra-1 (Fig. 5D). Consistent with the changes in morphology, inhibition of Fra-1 also reverted the EMT markers expression and higher migratory
behavior of SIRT1-overexpression cells to that seen in control cells (Fig. 5E and F). From these results, we can conclude that SIRT1 regulates EMT in a Fra-1-dependent manner.

**SIRT1 and Fra-1 predict poor survival of human colorectal cancer**

To investigate whether SIRT1 and Fra-1 expressions have clinical implications in human cancers, we examined their expressions in a tissue microarray containing 90 CRC and 90 paired non-CRC counterparts by IHC. A high expression level of SIRT1 was observed in 41.1% (37/90) of CRC tissues, whereas that was not observed in non-CRC counterparts (Table 1). A low expression level of SIRT1 was also observed in the rest of CRC tissues. These data suggested that SIRT1 expression in CRC tissues was significantly upregulated compared to that in non-CRC counterparts. Consistent with previous study [16], we found that SIRT1 protein was localized in both cytosol and nuclei of CRC cells (Fig. 6A). We further assessed the association between SIRT1 expression status and the clinical characteristics of CRC patients (Table 1). We found that SIRT1 expression was not related to gender, age, tumor size, and histological grade (Table 1), but it was significantly associated with TNM stage and Fra-1 expression status (Table 1, Fig. 6B). Likewise, Fra-1 expression was also significantly associated with TNM stage (Fig. 6B). Importantly, our data indicated that SIRT1 or Fra-1 overexpression correlated with worse overall survival in these CRC patients (Fig. 6C and D).
Discussion

Generally, metastasis is the main cause of death in patients with cancer. Despite ongoing research toward its underlying molecular mechanisms, specific determinants are widely unknown. Over the past few decades, growing evidence has indicated that SIRT1 may play a critical role in tumor initiation, progression, and drug resistance by blocking senescence and apoptosis, and promoting cell growth, angiogenesis and immune escape [10,11]. Importantly, several studies reported that SIRT1 overexpression correlated positively with more aggressive capability in patients with CRC, as well as in CRC cell lines [16,34]. Thus, we postulate that SIRT1 may be involved in CRC metastasis.

In this study, we studied the effect of SIRT1 on CRC metastasis in vitro and in vivo. Consistent with previous studies [16], our data showed there was a higher expression of SIRT1 in high–metastatic versus low-metastatic tumor cells. Moreover, ectopic expression of SIRT1 in low-metastatic cells markedly increased their metastatic capacity, whereas selective knockdown of high endogenous SIRT1 in high-metastatic cells reversed this capability. The in vivo xenograft mice experiments also revealed that knockdown of SIRT1 significantly reduced tumor metastasis. From these data, we can conclude that SIRT1 drives the migration and invasion in vitro and metastasis in vivo of CRC cells.

EMT is a very early event in which cancer cells switch to an aggressive phenotype [28]. It is now generally accepted that EMT plays a key role in tumor metastasis [25–27]. Our data showed that SIRT1 regulated EMT in CRC cells. This observation was supported by SIRT1 silencing in CRC cells with reverse EMT features, including a change in the expression of mesenchymal and epithelial markers. Consistent with our results, a previous study showed that inhibiting SIRT1 in human hepatocellular carcinoma impaired cell migration and invasion in vitro, as well as metastasis in vivo, which is significantly independent of the effects of SIRT1 on EMT [20]. The same effect was also observed in prostate cancer cells [21]. These findings strengthened the hypothesis that SIRT1 contributed to metastasis by regulating EMT.

Although most of the studies have demonstrated that SIRT1 promotes tumor progression in different types of cancer, some studies have shown that SIRT1 may function as tumor suppressor or metastasis suppressor gene. Kabra’s study showed that SIRT1 is an inhibitor of proliferation and tumor formation in colon cancer [35]. Simic et al. found that a decrease at SIRT1 level promoted metastasis via EMT in breast cancer [36]. Such discrepancy might be due to the difference of tumor types, downstream targets, and experimental setting, such as cell culture condition, and animal model.

It has been reported that Fra-1 is frequently elevated in a variety of human cancers and is strongly implicated in the tumor initiation and progression [37,38]. More recently, Fra-1 has been proposed as a gatekeeper of the EMT program during CRC metastasis [33]. Here, we found that SIRT1 regulated Fra-1 expression in CRC cells. Several previous studies reported that SIRT1 promoted transient and constitutive Wnt signaling in CRC cells [39,40]. Considering the view that Fra-1 is a key downstream target of Wnt signaling, SIRT1 may regulate Fra-1 expression by Wnt signaling. Since SIRT1 regulated Fra-1 and EMT, and the latter was also modulated by Fra-1, we wonder whether SIRT1 regulated EMT in a Fra-1-dependent manner.

Our data showed that inhibiting SIRT1 downregulated Fra-1 expression with concomitant MET, which is similar to directly silencing Fra-1. Furthermore, we found that the SIRT1-driven EMT phenotype, including the changes in morphology, the expression of mesenchymal and epithelial markers, and migration, was reverted after silencing Fra-1. These results strongly supported that SIRT1 contributed to EMT phenotype in a Fra-1-dependent manner.

The function of SIRT1 in driving cancer metastasis provides an intriguing explanation for the poorly understood progression and worse survival of cancer patients with aberrant SIRT1 expression. Here, we further demonstrated that SIRT1 overexpression in the clinical CRC samples was significantly correlated with TNM stage, metastasis. These data support a previous finding of which SIRT1 may play an important role in the progression of colorectal cancer [16]. Consistently, we also found that Fra-1 overexpression is associated with TNM stage, metastasis and SIRT1 expression, which further support the view that SIRT1 regulates Fra-1. In addition, our Kaplan–Meier survival analysis revealed that SIRT1 overexpression was significantly linked to worse overall survival, suggesting that SIRT1 can serve as a new predictor of prognosis in patients with CRC. Most importantly, combination of SIRT1 and Fra-1 status had a bigger predicted significance for prognosis.

In conclusion, we uncovered the mechanism as to how SIRT1 enhances the colorectal cancer cell migration and invasion via inducing EMT. Our findings provide conclusive evidence that reduction of SIRT1 has the potential to impair colorectal cancer cell migration and invasion in vitro and metastasis in vivo. This is followed by the attenuation of Fra-1 expression and EMT phenotype. Considering our sparse knowledge of the key events promoting tumor metastasis, this function of SIRT1 provides a rationale for clinically exploring the use of SIRT1 inhibitors in the therapy of metastatic CRC.

Acknowledgements

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Conflict of interest

No potential conflicts of interest were disclosed.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.03.010.
Fig. 6. SIRT1 and Fra-1 predict poor survival of human colorectal cancer (A) Expression of SIRT1 and Fra-1 was analyzed by IHC in the CRC tissues and paired non-CRC counterparts. (B) IHC analysis was performed to determine the SIRT1 and Fra-1 staining scores in CRC tissues with distinct TNM stage. *p < 0.05, **p < 0.01. (C and D) Kaplan-Meier curve for overall survival was compared according to SIRT1 or Fra-1 expression in CRC tissues.

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