Secreted Protein Acidic and Rich in Cysteines-Like 1 Suppresses Aggressiveness and Predicts Better Survival in Colorectal Cancers

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Running title: SPARCL1 predicts better survival of CRC.

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Abbreviations

SPARCL1: secreted protein acidic and rich in cysteines-like 1
CRC: colorectal cancer
ECM: extracellular matrix
ATCC: American Type Culture Collection
RT-PCR: reverse transcription-polymerase chain reaction
qRT-PCR: quantitative RT-PCR
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF: polyvinylidene fluoride
FFPE: formalin-fixed, paraffin-embedded
MTB: multiple tissue board
MTA: micro tissue array
IHC: immunohistochemistry
OR: odds ratio
95% CI: 95% confidence interval
HR: hazard ratio
OS: overall survival
H&E: Hematoxylin and Eosin
EMT: epithelial-mesenchymal transition
MET: mesenchymal-epithelial transition
Translational Relevance Statement

Secreted protein acidic and rich in cysteines-like 1 (SPARCL1) is an extracellular matrix glycoprotein with malignancy suppressing potential that is vital to the development of neural system. Here, our findings displayed that SPARCL1 inhibits cell proliferation, anchorage-independent growth and invasion, and induces differentiation in colon cancer cells. This study also explored the metastasis suppressing ability of SPARCL1 in a mouse xenograft model. Meanwhile, the invasion suppressing potential of SPARCL1 was assessed with outcome study based on two sets of colorectal cancer patients. The findings consistently validated that high expression of SPARCL1 was negatively associated with poor differentiation and metastasis, resulting in a better survival of colorectal cancers. Furthermore, we demonstrated that the SPARCL1 induced differentiation by mesenchymal-epithelial transition in colon cancer cells. These findings provided evidences for SPARCL1 as a potential prognostic biomarker, indicative of tumor invasion and metastasis of colorectal cancers.
Abstract

Background: Secreted protein acidic and rich in cysteines-like 1 (SPARCL1) is an extracellular matrix glycoprotein with malignancy suppressing potential. The hypothesis that SPARCL1 reduces cancer invasiveness and predicts better survival in colorectal cancers (CRC) was investigated.

Material and Methods: Stable SPARCL1 transfectants, RKO-SPARCL1, and corresponding vector control were constructed and implanted into nude mice to generate a mouse xenograft model of liver metastasis. Also, a retrospective outcome study was conducted on the COH set (222 CRCs) and ZJU set (412 CRCs). The protein expression level of SPARCL1 was determined by immunohistochemistry. The Kaplan-Meier and COX analyses were employed for survival analysis. The association of SPARCL1 with MET was examined by RT-PCR and Western blot analysis.

Results: The ectopic expression of SPARCL1 significantly reduced the potential for anchorage-independent growth, migration and invasion, and it induced cell differentiation in RKO and SW620 cells. In mouse xenograft model, the expression of SPARCL1 significantly reduced the liver metastasis ($P < 0.01$). The patient-based studies revealed that the expression of SPARCL1 was related to better differentiation ($P < 0.01$), less lymph node involvement (Odds Ratio, OR=0.67, 95% CI 0.45-1.00) and less distant metastasis (OR=0.38, 95% CI 0.18-0.79). The Kaplan-Meier and COX analysis demonstrated that the expression of SPARCL1 was associated with better overall survival (Log–rank $P < 0.01$; hazard ratio, HR=0.57, 95% CI 0.39-0.84). Transfection of SPARCL1 induced MET of colon cancer cells.
Conclusion: SPARCLI functions as a tumor suppressor promoting differentiation possibly via MET, which inhibits the aggressiveness of CRC.

Keywords: Secreted protein acidic and rich in cysteines-like 1 (SPARCLI), adenocarcinoma, colorectal cancer, prognostic biomarker, mesenchymal-epithelial transition.
Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer mortality in the world (1). Despite improvements in early detection and treatment of CRC in the last few decades, the 5-year survival rate remains about 50-60% (2, 3). This high mortality is partially attributable to liver metastasis which accounts for 40% to 50% of recurrences in CRC patients (4). Exploring the molecular basis of liver metastasis may provide further improvements in early detection, prevention, intervention and prognostic evaluation for patients with CRC.

Secreted protein acidic and rich in cysteines (SPARC) family includes SPARC, SPARCL1 and SMOC-2, etc. SPARCL1, which is also known as Hevin, SCI or MAST9, is an extracellular matrix (ECM) glycoprotein, whose gene is located in 4q22 (5, 6). SPARCL1 was first isolated from a human high endothelial venules cDNA library and is involved in many physiological functions, such as cell adhesion (7, 8), cell proliferation (7), central nervous system development (9) and B-lymphocyte maturation (10). SPARCL1 is widely expressed in normal tissues such as brain, heart, lung, muscle, colon and kidney (5, 6, 11-13). In contrast, its full-length expression was strongly down-regulated in several carcinomas such as metastatic prostate adenocarcinoma and non-small cell lung cancer (5, 8, 14-16). The down-regulation of SPARCL1 mRNA in colorectal carcinomas was confirmed by northern blotting (9) and oligonucleotide array (17). Previously, pilot studies demonstrated that high SPARCL1 expression level is associated with well differentiated status, less metastasis and better survival in CRCs (18). Similar results obtained by other investigators, which indicated that the expression of SPARCL1 increased with greater level of differentiation and decreased in
progressing from Dukes Stage B to D. However, their data showed that expression of
SPARCL1 led to poor survival in CRCs (19). Therefore, the prognostic value of
SPARCL1 needs to be further investigated at multiple centers with larger population.
Meanwhile, it was necessary to further explore the malignancy-suppressing ability of
SPARCL1 to clarify the mechanism(s).

In this study, we investigated whether the expression of SPARCL1 in colon cell
lines reduces cell proliferation, invasion or metastasis in an in vitro setting and in
mouse xenograft model. In addition, we explored the biological role of SPARCL1
protein in anchorage-independent growth, migration and differentiation of cells.
Furthermore, we conducted outcome studies on two CRC patient sets (COH set, 222
cases; and ZJU set, 412 cases) with different racial and socioeconomic backgrounds.
Two outcome studies yielded consistent findings indicating that the expression of
SPARCL1 predicts better survival in CRCs, implying that SPARCL1 may serve as a
potential prognostic biomarker for CRCs.
Materials and Methods

Cell culture and SPARCL1 expression plasmid transfection

Colon cancer cell lines (RKO and SW620) were obtained from American Type Culture Collection (ATCC, Manassas, VA). RKO was cultured in RPMI-1640 medium and SW620 cells in Leibovitz's L-15 medium at 37°C in 5% CO2. Culture medium was supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100U/ml) and streptomycin (100μg/ml). The human SPARCL1 cDNA was cloned into the pLXSN retroviral expression vector (Clontech, Mountain View, CA) and then transfected into the retroviral PT67 packaging cell line (Clontech, Mountain View, CA) using lipofectamine 2000 reagent (Invitrogen, San Diego, CA). After 72 hours incubation, virus-containing supernatants from PT67 cells were collected, and then filtrated by 0.45μm filters. The filtered supernatant was added to 70%-confluent cells in the presence of 8μg/ml polybrene (Sigma, St. Louise. MO). After 24 hours, cells were incubated with 500μg/ml of G418 for stable SPARCL1 expression transfectants selection. Meanwhile, the pLXSN vector was also transfected in the same way as a control.

Antibodies

Mouse monoclonal antibodies against SPARCL1 were produced by HuaAn Bio-Technology Co., Ltd (Hangzhou, China) using synthesized peptide (aa351-364: CDGPRHSASDDYFIP) as the antigen. The efficiency of antibodies was visualized by using recombinant human SPARCL1 protein (R&D, Minneapolis, MN) in Western blot analysis. The mouse monoclonal antibody IgG named No.C11 was pre-selected.
for further western blot and IHC staining experiments. In Western analysis, No.C11 significantly reduced non-specific signals in comparison with commercial antibody (Supplemental Fig 1). Moreover, the signal of recominant \textit{SPARCL1} protein and endogenous \textit{SPARCL1} (75 KDa and 130 KDa) could be specifically blocked by recominant \textit{SPARCL1} full length peptide (Supplemental Fig. 1), which indicated the specificity of antibody. The \textit{E-cadherin} (#3195), \textit{N-cadherin} (#4061) and \textit{Vimentin} (#3390) antibody were from Cell Signaling Technology (Boston, MA) and diluted as recommended. Anti-β-actin antibody was from Sigma and second antibodies were from Bio-Rad (Hercules, CA).

**Quantitative RT-PCR analysis and Western blot analysis**

RNA and protein were extracted from subconfluent cells in the exponential phase of growth. Total RNA was extracted and purified using Rneasy kit (Qiagen, Valencia, CA). Each mRNA sample (4μg) was reverse transcribed using the LongRange 2step RT-PCR Kit (Qiagen, Valencia, CA). An ABI One-Step Real-Time PCR System (Applied Biosystem, Mountain View, CA) was used for Quantitative RT-PCR (qRT-PCR) analysis. The experiments were performed in triplicate and normalized by β-actin (loading control). Primer sequences applied in qRT-PCR experiments are listed in supplemental table 1.

Secreted protein in cell culture supernatant was extracted using Trichloroacetic Acid-Acetone (Sigma) Precipitation. Protein in 20ml serum-free supernatant was resuspended in 20ul loading buffer. Cell lysate samples were extracted by M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) containing the protease.
inhibitor cocktail (Sigma). For Western blot analysis, about 40μg lysate samples or 20μl secreted protein were loaded into each well and separated by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to Polyvinylidene fluoride (PVDF) membrane. Immunoblotting was performed by incubation with Anti-SPARCL1 (No.C11, 1:200), E-cadherin, N-cadherin, Vimentin and β-actin antibodies as mentioned above then followed by incubation with corresponding alkaline phosphatase-conjugated second antibodies. Immunoreactive bands were visualized by enhanced-ECL or SuperSignal (Pierce, Rockfor, IL).

**In vitro cell growth assays**

The measurement of cell proliferation was based on cell counting on 24-well plates. At initiation of the study, approximately 1×10^4 cells were seeded per well. At each time point, cells on 24-well plates were trypsinized and then harvested. For cell counting, trypan blue staining was used to indicate viable cells. The proliferative activity was also determined by MTS assay (CellTilter 96TM Non-Radioactive Cell Proliferation Assay, Promega Corporation, WI), which monitors the number of viable cells after 4h in medium containing MTS. The conversion of MTS to formazan was measured in a plate reader at 490nm. The colony formation assay was used to reflect anchorage-independent cell growth. About 1×10^4 cells were layered in 60mm plate, the medium was replenished every five days and colonies that grew beyond 50μm in diameter after three weeks were scored as countable. Recombinant SPARCL1 protein (AF2728, R&D, CA) was added into the medium at the concentration of 1μg/μl. The mean and standard deviation were calculated from three independent experiments.
In vitro cell migration and invasion assay

Wound-healing assay was used to measure the ability of cell migration. 2\times10^5 viable cells were mixed thoroughly and seeded in 6-well plates. Twenty-four hours after seeding, the cell formed monolayer. A linear wound was created by scraping the cell monolayer with a 200\mu l sterile pipette tip. The media were carefully changed to remove any floating cells and cultured at 5% CO_2 and 37 °C for another 24 hours. To elucidate the role of SPARCL1 protein in wound healing, recombinant SPARCL1 protein was added into the medium at a concentration of 500ng/\mu l. Cells that spreaded into the scraped region were considered migrating cells. The ability of migration was represented by the migrating distance. To further validate the wound healing assay, Boyden-chamber (BD, Franklin Lakes, NJ) was employed with IL-8 as a chemoattractant at the concentration of 1\mu g/l \mu. A Matrigel chamber (BD, Franklin Lakes, NJ) was employed to evaluate the invasion ability of cancer cells. Assays were performed in 24-well plates with an 8\mu m polycarbonate filter membrane coated with growth-factor-reduced Matrigel diluted in 20%. Cells in serum-free medium (2.5\times10^4 cells per well) were added to the upper chambers. Conditioned medium with 10% FBS was placed in the lower chambers as a chemo-attractant. The chambers were incubated for 24 hours at 37°C with 5% CO_2. Migrated cells on the undersides of filter membrane were fixed in 90% (v/v) ethanol and stained with crystal violet. The migrated cells were counted using light microscopy and the experiments were performed in triplicate.

Construction of xenograft mouse models
All animal experiments were conducted under an approved protocol from the Institutional Animal Care and Use Committee (IACUC) of City of Hope. For subcutaneous xenograft mouse model, 15 6-week-old male athymic nude mice (Balb/cnu/nu) were divided into three groups: RKO (group 1), RKO-pLXSN (group 2) and RKO-SPARCLI (group 3). Five mice of each group were injected subcutaneously with 5×10^6 viable tumor cells at the site of costal region. Tumor size was measured every three days. Tumor volume was calculated based on length and width (20) and plotted against days post-injection. The mice were euthanized at the on Day 23 post-injection or when tumor diameter became larger than 1,000 mm^3. The fresh tumor specimen was divided into two parts. One part was fixed with 10% formalin and embedded in paraffin for histological examination; another part was snap-frozen and stored at -80°C for mRNA and protein extraction.

For liver-metastasis mouse model, 6-week-old male athymic nude mice (Balb/cnu/nu) were grouped as mentioned above. Under sterilization, mice were anesthetized with pentobarbital sodium. A transverse incision was made in the left flank to expose the spleen. Total of 5×10^6 viable tumor cells were injected into the spleen vein carefully to avoid extravasations. After five minutes post injection, the spleen was excised via ligation at the hilum of the spleen. The mice were euthanized at the Day 40 post-operation. Abdominal autopsy was performed to examine liver metastatic nodules. Histological examination was further committed by pathologists.

**Design of outcome study**

This is a retrospective population based outcome study. The design of study was
described on Supplemental Fig 2. The protocol of human subject application was reviewed and approved by the Institutional Review Board (IRB) of City of Hope (COH) (Duarte, CA, USA) and 2nd affiliated Hospital of Zhejiang University (ZJU) (Hangzhou, Zhejiang, China) respectively. All eligible participants received surgical operation and were diagnosed as colon or rectal adenocarcinoma. A total of 222 CRCs during 1980-2004 from COH (training set) and 412 CRCs during 1999-2006 from ZJU (validation set) were collected separately according to the following inclusion criteria: i) diagnosis of CRC by pathologist; ii) informed consent obtained; and iii) follow-up data available. The exclusion criteria include: i) no histological diagnosis; ii) informed consent unobtainable; iii) no follow-up data available; and iv) multiple cancers. The participants in the COH set included 185 Caucasian, 5 African-American, 13 Asian and 19 unknown ethnicity. All patients were Asian (Chinese) in the ZJU set. After surgical treatment, all patients were followed up until June 2007 for COH set, and September 2010 for ZJU set. The median follow-up period is 166 months in COH set and 46 months in ZJU set. All demographic data and pathological information were obtained by reviewing the hospital records (Supplemental Table 2). Follow-up data of COH set was obtained from cancer registry at COH. For ZJU set, it was obtained from a follow-up work team. Variables assessed included age, gender, date of operation, tumor location (colon or rectal), differentiation, tumor node metastasis (TNM) stage, adjuvant chemotherapy, radiotherapy, date of last follow-up and vital status at last follow-up. In ZJU, 88 patients were treated with chemotherapy and three with radiotherapy. For 222 cases in COH, 90 patients obtained post-surgery chemotherapy and 44 cases received radiotherapy. The chemotherapy regimen in both center is...
based on 5-Fu. The survival period was calculated from the date of surgical operation to the date patient was last seen. Only deaths from metastasis and local relapse of CRC were considered as disease-related death.

**Immunohistochemistry staining**

Formalin-fixed, paraffin-embedded (FFPE) cancer tissues from surgery were selected to construct multiple tissue board (MTB) for COH set, and multiple tissue array (MTA) for ZJU set (21). The condition of Immunohistochemistry (IHC) staining for SPARC LI was optimized on checkerboards with different normal and tumor samples. For each IHC staining, negative and positive checkerboards were included for quality control. Standard indirect immunoperoxidase procedures were used for immunohistochemistry. Briefly, 4μm-thick sections were cut from paraffin embedded tissue blocks, and immunostaining for each antigen was conducted using the avidin-biotin peroxidase complex technique (MaxVision™ HRP-Polymer IHC Kit, MAIXIN. Bio), following the manufacturer’s instructions. Anti-human SPARCLI monoclonal antibody (No.C11) (1:50 dilution) was used as the primary antibody. The slides resulting from the immunohistochemical reaction were evaluated individually and independently by two investigators in a double-blind manner. According to the percentages of positive cells and staining intensity, the immunohistochemistry result was assessed. Despite the heterogeneity of cancer cells, only cytoplasmic signal of SPARCLI was considered.

**Data management and statistical consideration**

MS-ACCESS was used to create a colorectal database. Double entries and logic correction were applied to reduce mistakes in the database. The JMP Statistical
Discovery Software (SAS Institute, Cary, NC) was employed for data analysis. The sample size estimation was calculated using nQuery Advisor 6.01 software. It was indicated that 200 cases would be sufficient for 80% study power with a two-sided $\alpha$ of 0.05. Multivariate logistic regression models were used to adjust for covariate effects on the odds ratio (OR). Kaplan-Meier survival analysis and Cox hazard proportional model were used for evaluation of overall survival (OS). ANOVA analysis was used for multiple comparisons. A $P$ value less than 0.05 was considered statistical significance.
Results

*SPARCL1* suppresses proliferation, migration and invasion of colon cancer cells

The mRNA level of *SPARCL1* was barely detectable in most of colorectal tumor cell lines including RKO and SW620 (Supplemental Fig. 3A). To investigate the role on malignancy, the *SPARCL1* expression plasmid (pLXSN-*SPARCL1*) and control vector (pLXSN) were transfected into RKO and SW620 cells. Stably expressing transfectants (RKO-*SPARCL1*, SW620-*SPARCL1* as well as the corresponding vector controls) were isolated. Western blot analyses confirmed that the protein level of *SPARCL1* was significantly higher in the expression transfectants in comparison to vector controls after normalizing to β-actin (Fig. 1A, *upper panels*). The secretion of *SPARCL1* protein was also elevated in the culture medium of *SPARCL1*-transfectant cells (Fig. 1A, *lower panels*). In the cell proliferation study performed using cell counting method, the growth of *SPARCL1* transfectants decreased significantly both in RKO (day 6, *P* = 0.03) and SW620 (day 6, *P* = 0.04) cells in comparison to corresponding vector control (Supplemental Fig. 3B *upper panels*). Additionally, when the MTS method was used to assess cell proliferation, it showed that the growth of *SPARCL1* transfectants decreased both in RKO (day 5, *P* > 0.05) and SW620 (day 5, *P* > 0.05) cells compare to the vector control (Supplemental Fig. 3B *lower panels*).

The ability of anchorage-independent growth also reflects the potential of cancer metastasis. Consistently, the colony formation of RKO-SPARCL1 and SW620-SPARCL1 was dropped by 54-62% (*P* < 0.01) and 19-48% (*P* = 0.03),
respectively, which indicated that anchorage-dependent growth was retarded in $SPARCL1$ expressing cells (Fig. 1B). Recombinant $SPARCL1$ peptide significantly reduced the colony formation by RKO cells ($P < 0.01$), as well as SW620 cells ($P < 0.01$) (Supplemental Fig. 3C).

The abilities to migrate and invade were regarded as the main malignant phenotype and prerequisite of metastasis for cancer cells. In wound-healing assay, it was shown that the $SPARCL1$ transfectants significantly reduced the migration ability of RKO cells (Fig. 1C, $P = 0.03$), as well as SW620 cells ($P = 0.03$). Recombinant $SPARCL1$ protein also inhibits cell migration (Supplemental Fig. 3D, $P = 0.03$ in RKO cells and $P > 0.05$ in SW620 cells). The Matrigel invasion assay demonstrated that the invasion ability of $SPARCL1$ transfectants decreased significantly (Fig. 1D, $P = 0.01$). The invasion ability was significantly enhanced when we applied IL-8 as chemoattractant in both RKO and SW620 cells ($P < 0.01$). While, it was significantly reduced by the recombinant $SPARCL1$ peptide ($P < 0.01$) (Supplemental Fig. 3E).

Above findings suggested that $SPARCL1$ could significantly reduce abilities of cell proliferation, anchorage-independent growth and invasion in colon cancer cell lines.

$SPARCL1$ inhibits tumor growth and liver metastasis in a mouse xenograft model

To validate the above findings, mouse xenograft models were employed to determine if $SPARCL1$ impacts tumor growth and metastasis $in vivo$. The RKO, RKO-pLXSN or RKO-$SPARCL1$ cells were implanted subcutaneously to form xenograft tumors. The tumor volume was monitored every three days. The average
tumor volume of RKO-SPARCL1 group was significantly reduced when compared to RKO group or RKO-pLXSN group (Supplemental Fig. 4). Through RT-PCR, the presence of human SPARCL1 in RKO-SPARCL1 xenografts was verified, but it was undetectable in RKO-pLXSN cells (Supplemental Fig. 4B). Representative gross views showed size disparity between RKO-pLXSN and RKO-SPARCL1 in subcutaneous xenograft mouse model (Supplemental Fig. 4C, left panels). The corresponding images for the hematoxylin and eosin (H&E) staining are also shown (Supplemental Fig. 4C, right panels). IHC staining confirmed that the SPARCL1 protein was expressed in the RKO-SPARCL1 xenograft group but absent in the RKO-pLXSN xenograft group (Supplemental Fig. 4D).

Since liver accounts for most of the CRC metastases, the impact of SPARCL1 on liver metastasis was assessed through intrasplenic implantation, a liver-metastasis model described in Materials and Methods. Laparotomy was performed on all the mice at 40 days after the implantation of tumor cells. It revealed that RKO-pLXSN cells formed on an average 98 metastatic nodules per liver in five mice analyzed as detected by dissection microscopy. In contrast, mice implanted with RKO-SPARCL1 cells formed on an average 11 nodules (Fig. 2A). It indicated that the metastatic nodules formation was significantly suppressed in SPARCL1 expressing transfectants (P < 0.01). The presence of human SPARCL1 in each implant tissue sample was determined by RT-PCR (Fig. 2B). Histological analyses confirmed that the number of micrometastatic lesions was markedly reduced in the livers of mice injected with SPARCL1 expressing cells (Fig. 2C). These results indicated that the expression of
SPARCL1 potentially inhibits liver metastasis of RKO cells in an orthotopic mouse model.

The SPARCL1 expression negatively associates with poor differentiation, lymph node involvement and distant organ metastasis in CRCs

To address whether SPARCL1 suppresses the invasiveness of cancers, the optimized IHC was applied to determine if the expression level of SPARCL1 was associated with the clinical features of CRC. All patients were enrolled at City of Hope (COH set, 222 cases) and Zhejiang University (ZJU set, 412 cases). The efficacy and specificity of SPARCL1 antibody was confirmed as described in Materials and Methods. Based on the expression level of SPARCL1 protein, all participants were divided into four subgroups: 0 (negative expression), 1 (weak positive), 2 (positive) and 3 (strong positive) (Supplemental Fig. 5). Alternately, 0 and 1 were re-defined as SPARCL1-low, while 2 and 3 were attributed to “SPARCL1-high”. In COH set, 65 of 222 CRC tissue samples were defined as SPARCL1-high expression; and in ZJU set, 182 out of 412 patients were regarded as SPARCL1-high.

Through IHC staining, higher expression of SPARCL1 was detected in well-differentiated cancers (Supplemental Fig. 5). Statistical analysis revealed that the percentage of SPARCL1-high increased with differentiation of CRCs in COH set ($P = 0.06$), and with statistical significance in ZJU set ($P < 0.01$) (Table 1). The statistical relevance between SPARCL1 and metastasis could not be determined in COH set ($P > 0.05$). While, a high level of SPARCL1 was negatively associated with lymph node involvement ($P = 0.049$) and distant organ metastasis ($P = 0.01$) in ZJU set (Table 1).
Further multivariate logistic analysis revealed that high level of *SPARCL1* expression was negatively related with lymph node involvement (Odds ratio, OR = 0.67, 95% CI 0.45-1.00) and distant metastasis (OR = 0.38, 95% CI 0.18-0.79) in ZJU set (Table 2).

Above findings suggested that the *SPARCL1* expression correlated with greater differentiation, and negatively associated with lymph node involvement and distant metastasis, which implies that *SPARCL1* may function as a tumor suppressor in CRCs.

**Higher *SPARCL1* expression is related to better prognosis in CRCs**

A retrospective outcome study was conducted on COH set (with 222 CRCs) as well as ZJU set (with 412 CRCs). In Kaplan-Meier analysis, higher expression of *SPARCL1* was associated with better survival in COH set (Fig. 3A, Log-rank *P*=0.05, univariate COX analysis *P*= 0.045). In ZJU set, overall survival was improved significantly in CRC patients with higher *SPARCL1* expression (Fig. 3B, Log-rank *P* < 0.01). Multivariate COX analysis was conducted to eliminate the confounder effects and further validate whether *SPARCL1* was associated with lower relative risk of death from CRC. The multivariate COX proportional hazard analysis demonstrated that high expression of *SPARCL1* represented the better survival of CRC (hazard ratio, HR = 0.33, 95% CI 0.33-1.05) in COH set (Fig. 3C). This finding also confirmed in ZJU set (HR = 0.57, 95% CI 0.39-0.84) (Fig. 3D). The HR’s pattern of other co-factors including metastasis, lymph node involvement, tumor invasion, differentiation, tumor location, gender and age are very similar, confirming the reliability of this study. To exclude the effect of chemotherapy, a multivariate COX analysis was conducted on patients without adjuvant chemotherapy or radiotherapy. It indicated that after
adjusting for age, gender, location, differentiation and TNM stage, HR of OS is 0.47 (95% CI 0.19-1.06) for COH set and 0.61 (95% CI 0.38-0.98) for ZJU set. These findings suggest that higher \textit{SPARCL1} expression in primary tumor leads to better survivability of CRC patients.

The stratification analysis was further employed to eliminate the confounders and explore whether \textit{SPARCL1} impacts prognosis differently in CRC patients with different TNM stages or tumor locations. For the colon cancer subgroup, the multivariate COX analyses revealed that \textit{SPARCL1} was related to better overall survival (HR = 0.63, 95% CI 0.33-1.13) in COH set (Supplemental Fig. 6A, left panel). The similar result could be seen in ZJU set (HR = 0.53, 95% CI 0.31-0.89) (Supplemental Fig. 6A right panel).

However, \textit{SPARCL1} seems not to impact the overall survival of rectal cancer patients in either COH set or ZJU set (Supplemental Table 3).

Further analysis of CRCs at different TNM stages indicated that \textit{SPARCL1} predicts better survival for both Stage I-II CRCs (without metastasis) and Stage III-IV CRCs (with metastasis). For stage I-II CRCs, Kaplan-Meier analysis indicated that \textit{SPARCL1} is associated with better survival in COH set (Log-rank \(P = 0.06\)) (Supplemental Fig. 6B left panel). It was consistent with results yielded from ZJU set (Log-rank \(P = 0.023\)) (Supplemental Fig. 6B, right panel). Meanwhile, \textit{SPARCL1} predicting better survival was also seen in CRC patients with stage III-IV (Supplemental Fig. 6C). Multivariate analysis also displayed similar results (Supplemental Table 3). It was indicated the \textit{SPARCL1} independently prognoses better survival of CRC with early or later stages.

The above findings demonstrated that \textit{SPARCL1} may serve as a potential...
prognostic biomarker and prognosticate better survival for CRC patients, especially for colon cancers.

**SPARCL1 relates to the differentiation of CRCs via MET**

To explore the mechanism of SPARCL1 in malignancy-suppression, the SPARCL1 and differentiation of CRC was investigated. The IHC staining showed the SPARCL1 expression in colon cancer section was eventually decrease in comparison with adjacent normal colon section(Fig 4A: a-c). Meanwhile, SPARCL1 steadily increased as with poor, moderate and well differentiation of CRCs (Fig 4A: d-f). The lumen-like formation indicated the ability to differentiate. An in vitro study demonstrated that up-regulation of SPARCL1 by gene transfection could obviously enhance the lumen-like formation in Matrigel in RKO and SW620 cells (Fig. 4B). Correspondingly, recombinant SPARCL1 peptide also could promote the lumen-like formation (Fig. 4). The E-cadherin, N-cadherin and Vimentin were regarding as the MET related genes. It was indicated that the mRNA expression level of E-cadherin was significantly up-regulated on RKO-SPARCL1 and SW620-SPARCL1 transfectants, and N-cadherin and Vimentin were significantly down-regulated (Fig. 4C). The Western blot analysis further confirmed that increase of that E-cadherin and decrease of N-cadherin and Vimentin could be seen in SPARCL1 expression RKO and SW620 transfectants (Fig. 4D). Meanwhile, qRT-PCR analysis also demonstrated that the differentiation-related genes GPNMB, NDRG1 and IGF1R were significantly up-regulated on RKO-SPARCL1 transfectants (Supplemental Fig. 4F). Above findings suggest that SPARCL1 induces differentiation through mesenchymal-epithelial
transition in colon cancer cells.
In this study, we demonstrated that *SPARCL1* suppresses the proliferation, migration, invasion and anchorage-independent growth of colon cancer cells (Fig. 1). The expression of *SPARCL1* also induces the differentiation of colon cancer cells (Fig. 4). The results are consistent with previously conducted *in vitro* studies (8, 22). Here, we further report the *in vivo* findings obtained using xenograft animal models. In the subcutaneous xenograft mouse model, the expression of *SPARCL1* retarded tumor growth, which points to its anti-proliferation potential (Supplemental Fig. 4). We utilized an intra-splenic injection mouse model mimicking liver metastasis of CRC at the later stages. This was based on the anatomy assumption that colon cancer cells migrate mostly through vena porta hepatic (23). The liver-metasis animal model indicated that *SPARCL1* significantly reduces the liver metastasis by RKO cells (Fig. 2). These *in vitro* and *in vivo* studies validate that the expression of *SPARCL1* has potential malignancy-suppressing ability in colon cancer cells.

In the outcomes study, expression of *SPARCL1* was significantly related to better survival of CRCs in two sets of patients with different socio-economic backgrounds. The *SPARCL1* expression was associated with well-differentiation in patients from COH set (*P* = 0.06) and ZJU set (*P* < 0.01), which was consistent with the *in vitro* studies. *SPARCL1* reduced the relative risk of lymph node involvement (OR = 0.66, 95% CI 0.45-0.99) and distant organ metastasis (OR = 0.38, 95% CI 0.17-0.79) in ZJU set. Besides the CRCs, down-regulation of *SPARCL1* was also reported for prostate...
and pancreatic cancers (22). Kaplan-Meier and COX proportional hazard analyses revealed that \textit{SPARCL1} functions as a protective factor. It prognosticates better survival of CRC patients both in COH set (HR = 0.33, 95% CI 0.33-1.05) and ZJU set (HR = 0.57, 95% CI 0.38-0.84). The protective effect of \textit{SPARCL1} could be observed in the ZJU set (Fig 3B, Supplemental Fig. 6B and 6C right panel). Whether \textit{SPARCL1} plays protective role in rectal cancers awaits further confirmation. A Swedish research team reported that \textit{SPARCL1} was positively associated with well-differentiation, which is consistent with our findings (19). However, their patient-based data indicated that \textit{SPARCL1} prognosticates poor survival in CRCs (19). The opposing results by two research teams may be due to different antibodies that were used for IHC. We tested the commercial antibody (R&D) and found many non-specific signals on Western blot analysis (Supplemental Fig 1). Therefore, we developed a new antibody (No. C11) and demonstrated that the nonspecific signals of No. C11 were barely seen on Western blot analysis (Supplemental Fig 1). Meanwhile, the intensity of specific signal detected by our antibody correlates with the \textit{SPARCL1} mRNA level (Fig 1A). The signal was blocked by \textit{SPARCL1} peptide (Supplemental Fig 1), indicating the reliability of our IHC staining technique.

The above evidence demonstrated the malignancy suppressing ability of \textit{SPARCL1}. Nevertheless, its function still remains largely unknown. In the previous studies, \textit{SPARCL1} was regarded as an astrocyte marker (24) and was proved to be essential in regulating cell-matrix interaction in the development of the brain (25). Increase in \textit{SPARCL1} was reported during the differentiation of embryonic stem cells into astrocytes (26). Our \textit{in vitro} experiment demonstrated that \textit{SPARCL1} induced the
ability of luminal like structure formation in Matrigel (Fig. 4B). It implied that the induction of differentiation could partly explain the malignancy-suppression by SPACRL1. The correlation and differentiation of SPARCL1 was also confirmed in a population-based study (Table 1).

SPARCL1 may function as a tumor suppressor by inducing differentiation. Our data indicated that SPARCL1 expression was increased as with well differentiation in CRC cancers. Colon cancer cells transfected with SPARCL1 or cultured with SPARCL1 peptide showed morphology diversity from the parental cell lines, which exhibited a luminal-like growth pattern. The E-cadherin was considered as epithelial marker; the N-cadherin and Vimentin were mesenchymal markers in epithelial-mesenchymal transition (MET) (34, 35). Our findings also suggested that SPARCL1 induced differentiation probably by MET. Both qRT-PCR and western blot analysis showed increase of E-cadherin and decrease of N-cadherin and Vimentin in SPARCL1 RKO and SW620 transfectants (Fig 4C and 4D). On other hands, previous studies demonstrated that GPNMB, NDRG1 and IGF1R were cell differentiation markers (27-29). GPNMB (Glycoprotein NMB, Osteoactivin) was reported to be an essential protein during the differentiation of osteoblast (30). N-Myc downstream-regulated gene 1 (NDRG1), also named differentiation-related gene-1, is involved in the development of central nervous system (31). NDRG1 was reported as a differentiation marker of breast cancer (32) and prognosticates better survival in CRCs(33). The IGF1R was considered as another differentiation related gene in lung adenocarcinoma (27). Here, the results indicated that the transcripts of GPNMB, NDRG1 and IGF1R were significantly increased in SPARCL1 transfectants.
Based on these studies, we inferred that loss of \textit{SPARCL1} in colon epithelial might lead to deficiency of differentiation and achievement of carcinogenesis by reducing MET. Therefore, induction of differentiation may, at least, be one of the pathways through which \textit{SPARCL1} suppresses malignancy in CRCs. \textit{SPARCL1} might be a potential differentiation marker for CRCs. Nevertheless, the detail mechanism of \textit{SPARCL1} during MET process needs to be further investigated.

Overall, the above findings revealed that \textit{SPARCL1} is a potential tumor suppressor gene by mediating cell differentiation, inhibiting proliferation ability and reducing cell invasion and metastasis potential. Thus, it relates to better prognosis in CRC patients.
Acknowledgements

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Figure Legend

Figure 1: SPARCL1 reversed malignant phenotypes and induced differentiation of colon cancer cells in vitro. A, Two SPARCL1 expression clones (RKO-SPARCL1 and SW620- SPARCL1) were created by transfecting with SPARCL1 expression plasmid pLXSN-SPARCL1, and vector control was constructed using pLXSN. The protein level of SPARCL1 was determined by RT-PCR and western blot with β-actin as a loading control. SPARCL1 protein in cell culture medium was also detected by western blot. B, Anchorage independent growth reduced in SPARCL1 expression cell line (SC1) compared to vector control (V) cells (*P = 0.03). Figures were demonstrated on right panel (scale bar, 100μm). C, Wound healing assay showed that SPARCL1 expression inhibited migration of RKO and SW620 cells after treatment for 24 hours (*P = 0.03). Representative result of RKO cell migration at 0 hours and 24 hours are on the right panels (Scale bar, 50μm). D, Relative invasion capacity of SPARCL1 expression cells (SC1) decreased compared with parental cell (PA) and vector control (V) in RKO and SW620 (*P < 0.01). The representative images of invasive cells were shown on right panel (Scale bar, 50μm).

Figure 2: Expression of SPARCL1 in RKO cells inhibited liver metastasis in xenograft mouse models. A, About 10^6 RKO-pLXSN or RKO-SPARCL1 cells were injected intrasplenic in Balb/cnu/nu nude mice through microsurgery. After operation, all mice were euthanized for examination on Day 40. The number of liver metastatic nodules was reduced in SPARCL1 transfectants (P <0.01). B, Expression levels of human SPARCL1 in liver metastatic nodules were detected by RT-PCR. C,
Representative photos of the livers (Scale bar, 1mm), H&E staining of SPARCL1 of metastatic tumor (M) and normal (N) liver tissues are shown (Scale bar, 50μm).

Figure 3: Higher expression level of SPARCL1 is related to better prognosis of CRCs. The SPARCL1 protein expression levels were scored based on density of IHC staining. The score 0 is negative staining, 1 is weak positive, 2 is positive and 3 is strong positive (Supplemental Fig. 5). To fit the COX analysis, the expression of SPARCL1 was re-categorized as SPARCL1-low (Score 0 and 1) and SPARCL1-high (Score 2 and 3). A, the Kaplan-Meier analysis for OS was displayed as SPARCL1-high vs SPARCL1-low to enhance the study power in COH set. B, the Kaplan-Meier analysis for OS was conducted in ZJU set. The multivariate COX analyses for OS of CRC were shown on C and D for COH set and ZJU set respectively. The hazard ration (HR) of SPARCL1 was based on SPARCL1-high vs. SPARCL1-low; Tumor invasion was T3-4 vs. T0-2; Lymphnode was positive vs. negative; Metastasis was yes vs. no; Differentiation was histological grade 2&3 vs. grade 1; Tumor location was rectum vs. colon; Gender was female vs. male; Age was based on per unit changes. The “*” was used to indicate statistical significance (P < 0.05).

Figure 4: SPARCL1 was related to differentiation of CRC via inducing MET. A, the IHC staining of SPARCL1 showed in adjacent normal colon epithelium(a), connection between normal and cancerous sections( b), and colon cancer section(c). The SPARCL1 expression increase as with grade with poor(d) , moderate(e) and well(f) differentiation, respectively. The scale bar represents 50μm in a, d, e and f, and
100μm in b; and 20μm in c. B, Colon cell formed luminal-like formation after transfected with SPARCLI or treated with recombinant SPARCLI protein (1μg/ul) (Scale bar, 50μm). C, Overexpression of SPARCLI caused changes of E-cadherin, N-cadherin and Vimentin in mRNA levels. The quantitative RT-PCR analysis showed down-regulation of N-cadherin and Vimentin, and up-regulation of E-cadherin in stable SPARCLI overexpression transfectants (*P < 0.01). D, the Western analysis showed the protein level of N-cadherin, Vimentin and E-cadherin. The GAPDH was used as a loading control.

References

6. Girard JP, Springer TA. Cloning from purified high endothelial venule cells of hevin, a close relative of the antiadhesive extracellular matrix protein SPARC.


Figure 1
Figure 3

A

COH set

Time from surgical operation (months)

Overall Survival

Case at risk

- SPARC1_Low 149
  - 65 41 18 4 1

- SPARC1_High 63
  - 38 23 11 3 1

HR=0.58
(95%CI 0.32-0.89)
Log-rank p=0.05

B

ZJU set

Time from surgical operation (months)

Overall Survival

Case at risk

- SPARC1(0) 65
  - 165 140 85 39 10 2 1

- SPARC1(1) 131
  - 118 86 40 21 4 1

- SPARC1(2) 51
  - 48 36 21 10 4 1

HR=0.57
(95%CI 0.39-0.83)
Log-rank p=0.01

C

COH set (N=222)

SPARC1
Metastasis
Lymphnode
Tumor invasion
Differentiation
Location
Gender
Age

HR (Range; 95% CI)(Log )

D

ZJU set (N=412)

SPARC1
Metastasis
Lymphnode
Tumor invasion
Differentiation
Location
Gender
Age

HR (Range; 95% CI)(Log )
Table 1. Pathoclinical characteristics and SPARCL1 distribution of eligible CRCs from COH and ZJU

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* SPARCL1-high includes positive and strong positive of cytoplasm staining score.
†† In ZJU set, there are 2 cases without data.
‡ Proximal colon includes: cecum, appendix, ascending colon, hepatic flexure, transverse and splenic flexure.
§ Distal colon includes: descending colon and sigmoid.
† In COH set, there are 10 cases without differentiation data.
** Statistical significance, p<0.05.
§§ According to the NCCN Clinical Practice Guidelines in Oncology™ Colon Cancer (V.3.2011) and Rectal Cancer (V.1.2011). In COH set, there are 8 cases without tumor invasion data and in ZJU set there are 3 cases without data.
### Table 2. Logistic analysis for SPARCL1 and TNM stages of CRCs

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Note: Logistic analysis was conducted to evaluate OR of SPARCL1 (high vs. low).
*Adjusted by gender and age.
†According to the NCCN Clinical Practice Guidelines in OncologyTM Colon Cancer (V.3.2011) and Rectal Cancer (V.3.2011). In COH set, there are 8 cases without tumor invasion data and in ZJU set there are 3 cases without data.

**Statistical significance, p<0.05
Clinical Cancer Research

Secreted Protein Acidic and Rich in Cysteines-Like 1 Suppresses Aggressiveness and Predicts Better Survival in Colorectal Cancers

Hanguang Hu, Hang Zhang, Weiting Ge, et al.

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