Original Article
LASP1 induces colorectal cancer proliferation and invasiveness through Hippo signaling and Nanog mediated EMT

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Abstract: The role of LIM and SH3 protein 1 (LASP1) in colorectal cancer (CRC) has been described in multiple studies, however, the underlying molecular mechanisms remained inclusive. In the present study, we performed immunohistochemistry (IHC) staining for LASP1 and found that LASP1 expression was higher in CRC tissue of advanced stage. Over-expressed (OE) LASP1 promoted proliferation, tumorigenesis and migration of CRC cell lines SW480 and SW620. Using the TCGA database, we identified Yes-associated protein (YAP1) was positively correlated with LASP1 expression in CRC patients. Introducing a novel YAP1 inhibitor CA3, we found that CA3 treatment inhibited LASP1 OE SW480 and SW620 cells proliferation, colony number formation, invasion and migration. Further mechanistic experiments showed that Nanog, a stem cell marker, was up-regulated in LASP1 OE cells but suppressed by CA3 treatment. Chromatin immunoprecipitation (CHIP) and luciferase reporter assay revealed that YAP1 can directly target the promoter region of Nanog and enhance its activity. LASP1 accelerated CRC migration through targeting YAP1-mediated vimentin and E-cadherin expression. Finally, by developing murine CRC model, we found the primary tumor size was almost abolished and the survival rate was greatly improved by chemotherapy and CA3 combined treatment compared with negative control or chemotherapy treated alone. Collectively, our findings demonstrated that LASP1 could induce CRC tumor cells proliferation and migration through activating hippo signaling pathway component YAP1 and further enhancing Nanog expression.

Keywords: LASP1, colorectal cancer, Hippo signaling pathway

Introduction

Colorectal cancer (CRC) is the most common diagnostic cancer and the second leading cause of cancer-related death worldwide [1]. Its burden is expected to increase to 60% with more than 2.2 million new cases and 1.1 million cancer deaths by 2030 [2]. Metastasis remains the main cause of CRC-related death and poor prognosis [3]. Currently, the underlying molecular mechanisms that trigger CRC tumor cells proliferation and metastasis are inclusive. Exploring key molecules that drive CRC progression might help to explore new potential therapeutic targets in clinic.

LIM and SH3 protein 1 (LASP1), was found up-regulated in several cancer types including breast cancer, esophageal squamous cell carcinoma, hepatocellular carcinoma, pancreatic cancer [4-7]. Although elevated LASP1 was reported in metastatic CRC tissues and associated with overall survive rate previously [8], its precise role in CRC was not known.

Hippo signaling pathway was implicated in the control of cancer cell-related unrestricted proliferation, invasiveness and metastasis [9, 10]. And its activity was best characterized by a YAP/TAZ transcriptional target signature of 22 genes [11]. Previous studies have reported that YAP was over-expressed in 52.5% cases of CRC tissues and associated with short overall survival [12]. The proliferation and invasion was promoted in YAP and TAZ over-expressed CRC cell line HCT116 [13, 14]. Nonetheless, how hippo signaling pathway was modulated in CRC remained to be fully elucidated.
In the present study, we found that LASP1 was highly expressed in late-stage CRC tissue and promoted colon cancer cells proliferation and invasiveness in vitro. Further mechanistic experiments revealed that LASP1 induced CRC progression through YAP1 mediated Nanog up-regulation and provided an innovative strategy in clinical CRC treatment.

Materials and methods

Cell lines and regents

The SW460 and SW620 cell lines used in the present study were obtained from ATCC (USA) and cultured in DMEM supplemented with 10% FBS (Hyclone, USA). CA3, CIS (cisplatin) and puromycin were purchased from Selleck (USA) and IgG was purchased from Solar-bio (China). Immunoprecipitation (IP) kit was purchased from BioVision (USA).

Tumor tissues and animals

CRC tissue samples were obtained from patients underwent surgery in the First Affiliated Hospital of Jinzhou Medical University from September 2018 to August 2019. Informed consent was obtained from all the patients. Patients subjected to chemotherapy, radiotherapy or combined with other tumors were excluded from this study. Nude mice were purchased from HFK Animal BIOSCIENCE Co., LTD (Beijing) and kept in compliance with the Guidelines for the First Affiliated Hospital of Jinzhou Medical University. Ethics were approved by the First Affiliated Hospital of Jinzhou Medical University.

Plasmid construction and transfection

A DNA fragment corresponding to human genomic LASP1 full-length DNA was cloned into pLVX-CMV-IRE5-Puro vectors (TAKARA, Japan). LASP1 CRISPR-Cas9 lentivirus and Nanog shRNA lentivirus were purchase from Sangon (China). Target cells were transfected with lentivirus and selected with 10 µg/mL puromycin for 7 days according to the manufacturers’ instructions. Transfection efficiency was determined by western blotting.

Immunohistochemistry and H&E staining

For immunohistochemistry, CRC tissues were fixed in 4% PFA overnight, dehydrated and embedded in paraffin. 3 µm tissue sections were sectioned and then blocked with 5% milk at room temperature for 30 minutes. Sections were stained with anti-LASP1 primary antibody (ab191022, Abcam, USA) overnight at 4°C, washed three times in PBS and followed by secondary antibody staining for 1 hour at room temperature (1:10000, Abcam, USA). Chromogenic revelation was performed using DAB Peroxidase Substrate Kit (1:50, Zsbio, China). For H&E staining, murine lung tissues were fixed in 37% formalin, embedded in paraffin and sectioned for subsequent haematoxylin & eosin staining. All the images were captured using a Zeiss microscope and the evaluation of staining intensities was calculated by ImageJ (National Institutes of Health, USA) according to the manufacturer’s protocol.

Cell proliferation assay

Cells were harvest, counted and seeded into 96-well plate. Cell proliferation was measured at 24, 48, and 72 h at 2.5×10³ cells per well. Cell proliferation assay was determined using the Cell Counting Kit-8 assay (Solar bio, China). Optical density was measured at 450 nm on Gen5 (BioTek, USA).

Colony-formation assay

Cells were harvest, counted and seeded into 6-well cell culture plate at the density of 2.5×10³ cells per well. After culturing at 37°C for 14 days, cells were fixed with 4% PFA (Solar bio, China), stained with crystal violet solution (Solar bio, China) and counted in each well.

Wound healing assay

Cells were seeded into 6-well cell culture plate at the density of 2x10⁶ cells per well. A sterile pipette was used to create wound on the cell layer and cells were cultured at 37°C for 24 h. Images of the scratch healing area were captured before and after incubation using a microscopy (Olympus Life Science, Japan).

Cell migration assays

2x10³ cells resuspended in 1 mL DMEM medium containing 1% FBS were seeded in the upper transwell chamber (Corning, USA). DMEM medium containing 20% FBS was added into the lower chamber. 4% PFA (Solar bio,
China) was used to fix cells transferred to the lower surface. Crystal violet solution (Solar bio, China) was used to stain cells.

**Western blotting**

Total proteins were extracted, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Solar bio, China) and transferred to a Hybond-NC membrane (Solar bio, China). After blocking with 5% milk, the membrane was incubated with primary antibody overnight at 4°C followed by incubation with secondary antibody (1:5000, Abcam, USA). Signals were detected using chemiluminescence (Tanon, China). Primary antibodies used for immunoblotting were listed as followings: LASP1 antibody (ab191022, Abcam, USA), YAP1 antibody (14074, CST), Epithelial-Mesenchymal Transition Antibody Sampler Kit (#9782, CST, USA), Nanog antibody (ab109250, Abcam), and β-actin antibody (8H10D10; CST, USA).

**Luciferase assay**

A DNA fragment corresponding to human genomic YAP1 full-length DNA and Nanog promoter DNA was cloned into pcDNA3.1 vector (Thermo, USA) and pGL3-Basic vectors (Promega, Japan), respectively. Cells were transfected with an appropriate reporter vector by means of Lipofectamine 2000. Luciferase activity was measured via a luciferase Assay Kit (Beyotime, China).

**ChIP assay**

The ChIP assay kit used in this experiment was purchased from Abcam (USA) and used as previously described [15]. 1 μg anti-YAP1 antibody was used in this experiment. Three pairs of primers flanking Nanog promoter were designed and the detailed sequence are shown in Table S1. All data were analyzed by 2-ΔΔCT method.

**RNA extraction and real-time PCR**

Total RNA was extracted using total RNA extraction kit for animal tissues (Tiagen, China) and 1 μg RNA was reversed into cDNA using one-step reverse transcription kit (TAKARA, Japan) following manufacturer's instructions. Real-time PCR was performed with an SYBR Premix (Solar bio, China) by Applied Biosystems 7500 Fast instrument (Thermo, USA). The primers are shown in Table S1. All data were analyzed using 2-ΔΔCT method.

**Animal tumor model**

1×10⁶ SW480 or SW620 cells were subcutaneously inoculated into mice to generate xenograft tumor model. The volume of transplanted tumors was measured every 10 days. For tumorigenesis assay, 5×10⁴ SW480 or SW620 cells infected with sgRNA virus or control virus were subcutaneously inoculated into mice. The number of tumors in each group was recorded at 21 day. For metastasis assay, 1×10⁶ SW480 or SW620 cells infected with lentivirus with different treatment were subcutaneously inoculated into mice. The number of liver metastasis tumors was recorded at 42 day.

**Statistical analysis**

SPSS 18.0 were used for statistical analysis. Descriptive statistical methods, student’s t tests, Kaplan-Meier diagrams, logarithmic rank tests, one-way analysis of variance and Spearman correlation tests were carried for statistical analysis when appropriate. *P<0.05, **P<0.01 and ***P<0.001 were considered statistically significant.

**Results**

**LASP1 facilitated tumor cells proliferation and invasion in CRC**

To explore whether LASP1 plays a role in CRC, we first set out to determine LASP1 expression in CRC tumor tissues. We performed IHC of collected CRC tumor tissue samples (n=20) and found that LASP1 expression was significantly higher in late stage (stage IV, n=10) than early stage (stage I, n=10) (Figure 1A, 1B). Next, we over-expressed or knockout LASP1 in CRC cell lines, SW480 and SW620, respectively. Successful over-expression or knockout lentivirus infection of LASP1 protein was determined by western blot (Figure 1C). We found that over-expression of LASP1 promoted cells proliferation while knockout of LASP1 strongly inhibited SW480 and SW620 cells growth determined by CCK8 assay (Figure 1D). LASP1 over-expression was found to decrease...
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Figure 1. LASP1 expression facilitated tumor growth and invasion in CRC. A. The immunohistochemistry of LASP1 in stage IV and stage I CRC tissues. B. Comparison of LASP1 expression in CRC tissue of stage IV and stage I, n=10. SW480 and SW620 cell lines were transfected with LASP1 OE lentivirus or LASP1 sgRNA lentivirus to establish LASP1 OE and LASP1 KO cells lines, respectively. C. The immunoblotting of LASP1 expression compared with negative control. D. Cell viability of LASP1 OE and LASP1 KO SW480 and SW620 cell lines was detected at various time points by CCK-8 assay. The result was normalized to the control group. E. Quantification of colonies formed by LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. The result was normalized to the control group. F. Relative migrant cell number of 1×10⁵ LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. The result was normalized to the control group. G. The wound-healing assay for evaluating the migration of LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. Gap width was normalized to the width at 0 h. H. H&E staining for hepatic metastasis in murine CRC model infected with negative control Cas9 or LASP1 sgRNA (n=3). The data was presented as the mean ± SEM from at least three independent experiments. Scale bar is 100 mm. *P<0.05; **P<0.01.
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and knockout was found to increase colony-formation efficiency in SW480 and SW620 cells, respectively (Figure 1E). Transwell and wound-healing assays showed that overexpression of LASP1 significantly enhanced and knockout of LASP1 inhibited CRC tumor cells migration and invasion (Figure 1F-H). Together, these results suggested that enhanced LASP1 expression in CRC tumor tissues might help promote CRC tumor cells proliferation and migration, which was associated with poor prognosis in CRC.

LASP1 promoted CRC progression via Hippo signaling pathway

Next, we set out to explore how LASP1 overexpression promoted CRC tumor cells proliferation and migration. In this regard, we examined the correlations of LASP1 and Hippo signaling pathway related genes, YAP1 in clinical cancer tissues. Using TCGA (https://tcga-data.nci.nih.gov/) database, we found that YAP1 was strongly positively correlated with LASP1 expression (COAD RNA-seq, n=457, Figure 2A), which suggested that LASP1 might promote tumor cell growth and metastasis via Hippo signaling pathway. The expression of YAP-1 was significantly reduced in LASP1 KO CRC cell lines but significantly enhanced in LASP1 OE SW480 and SW620 cells (Figure 2B). To further confirm the role of Hippo signaling pathway in LASP1 induced tumor proliferation and migration, a novel YAP1 inhibitor CA3, was applied to LASP1 OE CRC cells. Treatment with CA3 not only suppressed YAP1 expression, but also inhibited LASP1 induced tumor cells proliferation and colony number formation in SW480 and SW620 cells (Figure 2C-E). Moreover, inhibition of Hippo signaling pathway by CA3 also suppressed LASP1 OE CRC cells invasion confirmed by transwell and wound-healing assays (Figure 2F, 2G). Taken together, our findings suggested that LASP1 promoted CRC cells progression via activating Hippo signaling pathway.

LASP1 promoted CRC proliferation through targeting Yap1-mediated NANOG expression

To investigate the molecular mechanism of YAP-1 in LASP1 mediated CRC cells proliferation, we detected stem cells markers, NANOG, CD44, CD133, OCT4 and SOX2 by real-time PCR. The NANOG mRNA level was significantly inhibited by CA3 treated LASP1 OE SW480 and SW620 cells (Figure 3A). Moreover, knockout LASP1 significantly downregulated NANOG mRNA expression in SW480 and SW620 cells (Figure 3B). The above results suggested that LASP1 enhanced NANOG expression through YAP1. Next, we knockdown NANOG expression by shRNA lentivirus transfection and the knockdown efficiency was confirmed by western blot (Figure 3C). Nanog knockout significantly inhibited LASP1 OE cells proliferation as well as colony formation (Figure 3D, 3E). Furthermore, immunoblotting assay showed that Nanog expression was up-regulated in LASP1 OE cells but reversed by CA3 treatment, indicating that LASP1 enhanced Nanog promoter activity. Meanwhile, we performed Chromatin Immunoprecipitation (CHIP) assay to explore whether YAP1 can target Nanog promoter. As expected, LASP1-YAP1 axis significantly increased Nanog expression by increasing relative promoter binding and activity but reversed by CA3 treatment (Figure 3G, 3H).

LASP1 accelerated CRC migration through targeting YAP1 mediated epithelial-mesenchymal transition (EMT)

Since EMT is a well-known critical event in the progression toward cancer cells metastasis, we speculated that YAP1 take part in LASP1 mediated CRC proliferation through induction EMT occurrence. Firstly, by detecting Vimentin and E-cadherin expression via immunoblotting, we did observe enhanced Vimentin expression and impaired E-cadherin expression in LASP1 OE SW480 and SW620 cells (Figure 2C-E). Moreover, inhibition of Hippo signaling pathway by CA3 also suppressed LASP1 OE CRC cells invasion confirmed by transwell and wound-healing assays (Figure 2F, 2G). Taken together, our findings suggested that LASP1 promoted CRC cells progression via activating Hippo signaling pathway.

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Figure 2. LASP1 induced CRC tumor cells proliferation and migration through the activation of YAP1. A. Correlation analysis of YAP1 expression with LASP1 using the TCGA (https://tcga-data.nci.nih.gov/) database. B. YAP1 expression in SW480-LASP1 KO and SW620-LASP1 KO cell lines or LASP1 OE cell lines was analyzed by western blotting. C. YAP1 expression in SW480-vector, SW480-LASP1 OE, SW480-LASP1 OE treated with CA3 (1 µM), SW620-vector, SW620-LASP1 OE and SW620-LASP1 OE treated with CA3 (1 µM) was analyzed by western blotting. D. Cell viability of LASP1 OE, CA3 (1 µM)-treated LASP1 OE SW480 and SW620 cell lines at various time points by CCK-8 assay. The result was normalized to the control group. E. Quantification of colonies formed in LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. The result was normalized to the control group. F. Relative migrant cell number of 1×10^5 LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. The result was normalized to the control group. G. The wound-healing assay for evaluating the migration of LASP1 OE and LASP1 KO SW480 and SW620 cell lines for 24 h. Gap width was normalized to the width at 0 h. The data was presented as the mean ± SEM from at least three independent experiments. Scale bar is 100 mm. *P<0.05; **P<0.01.
Figure 3. LASP1 promoted the proliferation of CRC tumor cells through YAP1-mediated up-regulation of Nanog. The relative expression of stem cell markers, NANOG, CD44, CD133, OCT and SOX2 in (A) LASP1 OE and CA3 (1 µM) treated LASP1 OE SW480 and SW620 cells and (B) LASP1 KO SW480 and SW620 cells measured by real-time PCR. LASP1 OE SW480 and SW620 cell lines infected with Nanog shRNA lentivirus. (C) Western blotting analysis of Nanog expression compared with negative control. (D) Cell viability at various time points by CCK-8 assay. The result was normalized to the control group. (E) Quantification of colonies formed for 24 h. The result was normalized to the control group. (F) Western blotting analysis of Nanog expression. Luciferase assay was performed by transfecting pGL3-Human Nanog promoter vector + NC vector (NC group), pGL3-Human Nanog promoter vector + YAP1 expression vectors (OE group) or pGL3-Human Nanog promoter vector + YAP1 expression vectors + CA3 (10 µM) (CA3 group) into cells. (G) Relative luciferase activity in SW480-vector, SW480-LASP1 OE, SW480-LASP1 OE treated with CA3 (1 µM), SW620-vector, SW620-LASP1 OE and SW620-LASP1 OE treated with CA3 (1 µM). The result was normalized to the vector (PGL3.0) group. (H) Relative promoter enrichment in LASP1 OE and CA3 (1 µM) treated LASP1 OE SW480 and SW620 cell lines in CHIP assay. The result was normalized to the IgG group. The data was presented as the mean ± SEM from at least three independent experiments. *P<0.05; **P<0.01.
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As expected, knockout of NANOG also suppressed liver metastases in murine CRC model (Figure 4E, 4F). Collectively, these results suggested that YAP1 enhanced Nanog expression might play a critical role in LASP1 mediated CRC invasion.

Finally, we injected LASP1 OE or KO SW460 or SW620 cell lines to nude mice and confirmed that LASP1 could promote tumorigenesis in 4D. YAP-1 mediated Hippo signaling pathway regulated LASP1 promoted xenograft mouse model.
vivo (Figure 5A). CA3 treatment or knockout of Nanog compromised LASP1 induced tumorigenesis (Figure 5B, 5C). To further evaluate the association between LASP1 and YAP1, we evaluated tumor volume and survival rate by different treatment in LASP1 OE cells induced murine CRC model. The size of primary tumor was almost abolished by CIS + CA3/5-FU + CA3 treatment compared with negative control or CIS/5-FU treatment alone (Figure 5D). Accordingly, the survival rate greatly improved in CIS + CA3/5-FU + CA3 treated group (Figure 5E). Taken together, these observations implicated that YAP1 is a critical regulator of LASP1 induced CRC aggressiveness in vivo.

Discussion

Our present study investigated the molecular mechanisms of how LASP1 promoted CRC progression and metastasis. Our data confirmed higher levels of LASP1 in CRC tissues in late stage than that in early stage. Overexpression of LASP1 significantly promoted CRC tumor...
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...cells proliferation, progression and metastasis in vitro and in vivo. Hippo signaling pathway, characterized by YAP1, was found to play an important role in LASP1 mediated CRC proliferation and migration confirmed by in vitro and in vivo studies. Furthermore, a stem cell marker Nanog, was found to be regulated by YAP1 and play a role in LASP1 induced CRC tumor cells proliferation, tumorigenesis as well as EMT occurrence in our present study.

The roles of LASP1 and hippo signaling pathway in promoting CRC tumor cells proliferation and migration were reported previously. Niu et al found that LASP1 promoted CRC aggressiveness by forming LASP1-S100A11 axis to modulate TGF-beta/Smad signaling pathway. They also identified flotillin-1 (FLOT1) and histone H1 as critical factors to modulate LASP1-S100A11 axis-mediated EMT. The involvement of LASP1 in promoting CRC progression by activating PI3K/AKT signaling pathway was also determined [16]. Considered as an important regulator of cell growth, proliferation and apoptosis, hippo signaling pathway plays a critical role in suppressing tumor growth and metastasis [17]. YAP1 abnormal activation was associated with poor prognosis in various human cancers [18]. Niu et al discovered that RASAL2, encoding a RAS-GTPase-activating protein (RAS-GAP), promoted CRC progression through LATS2/YAP1 axis [19]. FGF8, which belongs to the human fibroblast growth factor (FGF) family and TEAD4 (TEA domain family member 4) was reported to promote CRC tumorigenesis and metastasis via transcriptionally targeting YAP1 [20, 21]. Our present study demonstrated that LASP1 promoted CRC progression by targeting hippo signaling pathway. GEO database analysis indicated a correlation between LASP1 and YAP1. Further in vitro and in vivo study demonstrated that inhibition of Hippo signaling pathway by CA3 suppressed LASP1 OE CRC tumor cells growth, tumorigenesis and metastasis. Our study elucidated a new mechanism that LASP1 accelerated CRC tumor cells proliferation and migration partly through activating Hippo signaling pathway.

NANOG is a homebox transcription factor that plays a significant role in maintaining pluripotency and self-renewal of both embryonic stem cells and adult stem cells. It was also reported to be involved in the tumorigenesis and metastasis of CRC [22]. Previous study found increased NANOG expression with statistically associated with more advanced stage of the tumor. They demonstrated that wnt-signaling component and nuclear b-catenin/TCF4: c-JUN complex is a key nuclear mediator for NANOG1 activity [23]. Meng et al demonstrated that Nanog, acted as an inducer of EMT related signals in CRC, could be regulated by snail and initiated by TGF-β1 [24]. Our present study found that in CRC, Nanog could also be regulated by hippo signaling pathway component, YAP1 and might play a critical role in LASP1 mediated CRC malignancy.

**Conclusions**

In summary, our present study demonstrated a novel mechanism of LASP1 induced CRC progressing by activating hippo signaling pathway. YAP1, an important component of hippo-signaling, could regulate CRC tumor cells proliferation and migration through upregulating Nanog expression. Further study will focus more on the role of LAPS1-YAP1-Nanog axis in drug resistance of CRC.

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**Disclosure of conflict of interest**

None.

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**References**


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### Table S1. Sequences of primers for Real-time PCR

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<th>Gene</th>
<th>5’ Primer</th>
<th>3’ Primer</th>
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