SRC-like adaptor protein negatively regulates Wnt signaling in intrahepatic cholangiocarcinoma

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Received January 10, 2018; Accepted June 29, 2018

DOI: 10.3892/ol.2019.9901

Abstract. Currently, the molecular mechanisms underlying intrahepatic cholangiocarcinoma (IHCC) are poorly understood. In the present study, the focus was primarily on SRC-like adaptor protein (SLAP), an adaptor protein, which is aberrantly expressed in various cancer types. To the best of our knowledge, the present study was the first to demonstrate that SLAP was decreased in IHCC tissues and cells, compared with controls. Further study indicated that SLAP overexpression suppressed IHCC cell proliferation and induced cell cycle arrest, indicating the tumor suppressor role of SLAP in IHCC progression. To demonstrate the effects of SLAP on Wnt signaling, the β-catenin/T cell factor transcription reporter assay was conducted. Compared with the negative adenovirus vector control (Ad-NC), overexpression of SLAP reduced TOPFlash activity, and no changes in FOPFlash activity were identified. Furthermore, the expression levels of Wnt target genes, including β-catenin, c-Myc, cluster of differentiation 44, Slug, Vimentin and matrix metallopeptidase-9, were reduced in RBE and Huh28 cells overexpressing SLAP. Additionally, the effects of SLAP on IHCC cell invasion and migration were determined. Compared with the Ad-NC control, the migration and invasion capacity was reduced following overexpression of SLAP in RBE and Huh28 cells. In summary, reduced SLAP expression may enhance IHCC malignant progression by activating Wnt signaling.

Introduction

Intrahepatic cholangiocarcinoma (IHCC) is the second most common hepatic malignancy worldwide (1,2) and its typical characteristic feature is abnormal biliary epithelial differentiation (1,2). It is highly aggressive due to early invasion, widespread metastasis and the lack of therapeutic strategies (3,4); therefore, it is important to determine the molecular mechanisms underlying IHCC to facilitate the development of novel diagnostic and therapeutic approaches, in order to improve the treatment of IHCC.

Numerous cellular processes are regulated by extracellular factors (5,6). For instance, extracellular factors, including inflammation factors and cytokines, regulate signaling of various cell surface receptors, including the B cell receptor, the T cell receptor, cytokine receptors and receptor tyrosine kinases, which serve key roles in immune and cancer cell signaling (6). In order to transduce signals, extracellular factors bind to cell surface receptors and regulate intracellular signaling molecules through adaptor proteins. Adaptor proteins contain a multitude of functional domains and facilitate signal transduction by forming multi-protein complexes (7). SRC-like adaptor protein (SLAP) has SRC-homology 2 (SH2) and SH3 domains (8). Using its SH2 domain, SLAP can interact with a number of receptors through phosphotyrosine residues and facilitate the impairment of Src-mediated signaling by competing with Src as it binds to phosphotyrosine residues (7). SLAP is expressed in a variety of tissues and controls the downstream signaling by binding to various receptors (6). Through recruiting E3 ubiquitin ligase Cbl, SLAP inversely regulates various receptor-signaling pathways via an unknown mechanism (9).

The Wnt/β-catenin pathway serves a key role in IHCC cell growth, metastasis and cancer susceptibility (10,11). β-catenin acts as an important co-activator of Wnt-mediated gene expression (12). Following the presence of Wnt ligands, β-catenin accumulates in the cytoplasm and is transported to the nucleus, where it combines with the lymphocyte enhancer factor (LEF)/T cell factor (TCF) complex, in order to recruit chromatin remodeling complexes and activate gene expression (13). β-catenin binding to upstream promoter regions activates the expression of various genes involved in proliferation, such as c-Myc and cyclin D1, and metastasis, such as matrix metallopeptidase-9 (MMP-9) (14). c-Myc and cyclin D1 are key proto-oncogenes with similar downstream effects. c-Myc has multiple putative targets, including genes involved in cell cycle control, apoptosis, DNA metabolism and dynamics, energy metabolism and macromolecular synthesis (15).
Additionally, cyclin D1 serves key roles in cell cycle progression in the transition from G1/G0 to S phase (16). MMP-9 is an extracellular matrix-degrading enzyme that is involved in the initiation of cell invasion and migration, and is capable of degrading type V, VII and X collagen (17). Upregulation of MMP-9 may destroy the integrity of the basement membrane and further increase tumor cell invasion and migration through the basement membrane structure (18); however, the association between SLAP and the Wnt/β-catenin pathway is poorly understood.

In the present study, it was demonstrated that SLAP expression was decreased in IHCC tissues compared with adjacent non-cancer tissues. Further study indicated that SLAP expression is inversely associated with the activation of Wnt/β-catenin signaling, thereby contributing to the malignancy and progression of IHCC.

Materials and methods

Cell culture. Human intrahepatic biliary epithelial cell line HIBEpiC (UFI10957; Shanghai Junrui Bio Tech., Shanghai, China, http://junrui.shengwu.bioon.com.cn/), and human IHCC cell lines HuCCT1 (American Type Culture Collection, Manassas, VA, USA), HCCC-9810 (Nanjing KeyGen Biotech Co., Ltd.), RBE (Nanjing KeyGen Biotech Co., Ltd.) and Huh28 (Nanjing Keygen Biotech Co., Ltd.) were cultured at 37°C in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal calf serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (HyClone; GE Healthcare Life Sciences) in humidified atmosphere containing 5% CO₂.

Patient samples. Samples, including 30 primary IHCCs and 30 adjacent non-cancerous liver tissues containing normal intrahepatic bile ducts (at least 5 cm from the tumor edge), were obtained from the Department of Hepatobiliary and Pancreatic Surgery, Xiangya Hospital, Central South University between January 2016 and January 2017 (Changsha, China). The patients’ characteristics are summarized in Table I. The study was approved by the Ethics Committee of Xiangya Hospital, as stipulated by the Declaration of Helsinki, with written informed consent for the use of the specimens from all enrolled patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was isolated from primary IHCCs and 30 adjacent non-cancerous liver tissues or HuCCT1 cells using RNAzol (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China), according to the manufacturer's protocol. The concentration and the purity of RNA samples was determined by measuring the optical density (OD) 260/280. A total of 1 µg RNA was reverse transcribed using Moloney Murine Leukemia Virus reverse transcription enzyme (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with specific primers. The temperature protocol used for RT was as follows: 72°C for 10 min; 42°C for 60 min; 72°C for 5 min and 95°C for 2 min. To quantify the relative mRNA expression levels, qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in an iCycleriQ real-time PCR detection system. The PCR amplifications were performed in a 10 µl reaction system containing 5 µl SYBR Green Supermix, 0.4 µl forward primer, 0.4 µl reverse primer, 2.2 µl double distilled H₂O and 2 µl template cDNA. Thermocycling conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The RT-qPCR primers were as follows: SLAP, forward, 5'-TGGCTGGATCGGGTAGTAA-3', and reverse, 5'-CCCCATTTTCTGAGCTG-3'; and GAPDH, forward, 5'-AACGGGAAGCTTGTCAATGGAA-3', and reverse, 5'-GATACGCAAGGGGGCGAC-3'. GAPDH served as an internal control. Experiments were repeated three times in duplicates. The relative gene expression was calculated using the 2-ΔΔCq method (19).

Construction of adenoviral vectors. Adenoviral vectors overexpressing SLAP (Ad-SLAP) or negative control (NC) (Ad-NC) (contract no. GCPA87909) were constructed by Shanghai GeneChem Co., Ltd. (Shanghai, China). For transfection, 10⁶ cells/well were seeded in six-well plate. After 24 h, Ad-SLAP and Ad-NC was transfected into six-well plate at 50 multiples of infection (MOI) for 48 h. Subsequently, the cells were collected for further analysis.

Cell proliferation and cell cycle assays. The Cell Counting kit-8 (CCK-8; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was used to determine cell proliferation. Cells transfected with Ad-SLAP or Ad-NC were seeded into 96-well plates at 2,000 cells/well. Briefly, 10 µl CCK-8 solution was added into each well after 1, 2, 3, 4 and 5 days incubation at 37°C for proliferation measurement. In viable cells, WST-8 was metabolized, producing a chromogen that was detected at 450 nm using a Spectra Max M2 spectrophotometer (SpectraMax M2; Molecular Devices, LLC, Sunnyvale, CA, USA).

For cell cycle analysis, transfected cells were harvested after 48 h and fixed with 70% ethanol at -20°C for 24 h. Subsequently, HIBEpiC cells (~1x10⁶) cells were trypsinized, washed twice with PBS and fixed in 70% ice-cold ethanol for 1 h at 0°C. The samples were centrifuged at 300 x g for 5 min at 4°C, the ethanol removed and they were exposed to 100 mg/ml RNaseA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 30 min at 37°C. Cellular DNA was stained with propidium iodide at 37°C for 15 min (Nanjing Keygen Biotech Co., Ltd.). Cell-cycle distributions were determined by flow cytometry using a BD FACSCalibur system (BD Biosciences, Franklin Lakes, NJ, USA) and data were analyzed using the ModFit software 4.1 (Verity Software House, Inc., Topsham, ME, USA).

Colony formation assay. For a colony formation assay, 500 cells/well were seeded in 6-well plates, transfected with si-SLAP or NC and cultured for 2 weeks in RPMI-1640 medium at 37°C. Colonies with >50 cells were counted and fixed with 4% paraformaldehyde for 15 min at room temperature. The colonies were fixed with 90% methanol for 15 min at room temperature and stained with giemsa dye solution (Beijing Solarbio Science & Technology Co., Ltd.) for 10 min at room temperature. All experiments were performed in triplicate wells and repeated at least three times. The photographs were obtained under a light microscope (magnification, x40).
Table I. Clinicopathological features of patients with IHCC.

| Clinicopathological features | Patients with IHCC, n |
|-----------------------------|-----------------------|
| Total                       | 30                    |
| Sex                         |                       |
| Male                        | 20                    |
| Female                      | 10                    |
| Age, years                  |                       |
| ≥60                         | 18                    |
| <60                         | 12                    |
| Degree of differentiationa |                       |
| Good                        | 5                     |
| Moderate                    | 18                    |
| Poor                        | 7                     |
| Tumor size, cm              |                       |
| ≤4.0                        | 16                    |
| >4.0                        | 14                    |
| CA199                       |                       |
| ≤1 kU/l                     | 18                    |
| >35 kU/l                    | 12                    |
| Lymph node metastasis       |                       |
| No                          | 13                    |
| Yes                         | 17                    |
| TNM stage                   |                       |
| I and II                    | 6                     |
| III                         | 18                    |
| IV                          | 6                     |

TNM, Tumor-Node-Metastasis; IHCC, intrahepatic cholangiocarcinoma. *According to World Health Organization classification.

β-catenin/TCF transcription reporter assay. Briefly, 1x105 cells/well were seeded in a 24-well plate in RPMI-1640 medium at 37°C for 24 h prior to transfection with the TOPFlash or FOPFlash reporter plasmids (EMD Millipore, Billerica, MA, USA). For transfection, 0.8 μg TOPFlash or FOPFlash plasmid were mixed with 2 μl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocols. Additionally, the cells were co-transfected with 0.02 μg of an internal control reporter plasmid for Renilla reniformis luciferase (Promega Corporation, Madison, WI, USA) expression driven by the tyrosine kinase promoter, in order to monitor the transfection efficiency in reporter assays. After transfection for 24 h, a dual luciferase reporter assay was carried out with the Dual Luciferase Assay System kit (Promega Corporation). Relative luciferase units were used to calculate the fold-induction normalized to Renilla reniformis luciferase expression for transfection efficiency. The relative luciferase activity was determined using a Promega GloMax 20/20 luminescence detector (Promega Corporation).

Western blotting. Cell extracts were collected using the radio-immunoprecipitation assay buffer (1% TritonX-100, 15 mmol/l NaCl, 5 mmol/l EDTA and 10 mmol/l Tris-HCl; pH 7.0; Beijing Solarbio Science & Technology Co., Ltd.) supplemented with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA.) A bicinchoninic protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration, according to the manufacturer's protocols. Equal quantities of protein (15 μg) were separated in the 10% SDS-PAGE, followed by transfer of electrophoresed proteins onto nitrocellulose membranes. The membranes were blocked in 8% nonfat milk at room temperature for 2 h and incubated with primary antibodies against β-catenin (dilution, 1:1,000; cat. no. ab32572; Abcam, Cambridge, UK), vimentin (dilution, 1:1,000; cat. no. ab92547; Abcam), MMP-9 (dilution, 1:1,000; cat. no. ab73734; Abcam), and β-actin (dilution, 1:5,000; cat. no. ab8226; Abcam) at 4°C overnight. Following several washes with TBST, the membranes were incubated with horseradish-peroxidase (HRP)-conjugated goat anti-rabbit (dilution, 1:5,000; cat. no. ZF-0311; Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd., Beijing, China) for 2 h at room temperature and subsequently washed. Immunodetection was performed using an enhanced chemiluminescence detection system (EMD Millipore), according to the manufacturer's protocol. The house-keeping gene β-actin was used as the internal control. ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA) was used for density analysis.

Statistical analysis. The data are presented as means ± standard deviation from three independent experiments. Paired Student's t-tests were used for comparisons of two groups. One way analysis of variance (SPSS 13.0; SPSS, Inc., Chicago, IL, USA) was used for multiple comparisons followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.
Results

* Decreased SLAP expression in IHCC tissues and cell lines. Compared with adjacent non-cancerous tissues, the SLAP expression was significantly decreased in IHCC cancer tissues (P<0.05; Fig. 1A). The mRNA level of SLAP was also compared with clinical data associated with the degree of tumor malignancy, including lymph node metastasis and tumor size. As depicted in Fig. 1B, the mRNA level of SLAP was notably higher in patients with IHCC with lymph node metastasis (1.00±0.48), compared with those without lymph node metastasis (0.41±0.30) (P<0.05). Furthermore,
the mRNA level of SLAP was decreased in patients with IHCC with tumor size ≤4.0 cm (0.47±0.23), compared with those with tumor size >4.0 cm (1.00±0.70) (P<0.05; Fig. 1C). These data indicated that decreased SLAP level enhanced the malignancy of IHCC. Additionally, the SLAP expression was evaluated in human intrahepatic biliary epithelial cell line HIBEpiC and human IHCC cell lines HuCCT1, HCCC-9810, RBE and Huh28. High expression levels of

Figure 2. SLAP overexpression suppresses IHCC cell proliferation and induces cell cycle arrest. (A) The protein expression level of SLAP was significantly upregulated in RBE (left side) and Huh28 (right side) cells following transfection with Ad-SLAP, compared with Ad-NC-transfected cells. (B) The Cell Counting Kit-8 assay demonstrated a slower growth of IHCC cells transfected with Ad-SLAP, compared with cells transfected with Ad-NC at 1, 2, 3, 4 and 5 days. (C) The flow cytometry analysis indicated that SLAP overexpression induced cell cycle arrest in RBE and Huh28 cells compared with the Ad-NC-transfected cells. *P<0.05, **P<0.01 and ***P<0.001 vs. control. SLAP, SRC-like adaptor protein; IHCC, intrahepatic cholangiocarcinoma; NC, negative control.
SLAP were identified in HIBEpiC cells (Fig. 1D); however, the protein expression of SLAP was decreased in HuCCT1, HCCC-9810, RBE and Huh28 cells (P<0.05 and P<0.001; Fig. 1D). Due to the expression of SLAP being the lowest in RBE and Huh28 cells, these two cell lines were selected for further study.

SLAP overexpression inhibits IHCC cell proliferation and induces cell cycle arrest. To overexpress SLAP, RBE and Huh28 cells were transfected with Ad-SLAP for 1, 2 or 3 days. As depicted in Fig. 2A, the protein level of SLAP was significantly upregulated in RBE and Huh28 cells. The CCK-8 assay demonstrated a slower growth of IHCC cells transfected...
with Ad-SLAP, compared with Ad-NC at 1, 2, 3, 4 and 5 days (P<0.05, P<0.01, P<0.001, t-test) (Fig. 2B). Flow cytometry analysis indicated that overexpression of SLAP induced a more pronounced cell cycle arrest in RBE and Huh28 cells,
compared with the control group (P<0.05, P<0.01, t-test) (Fig. 2C). These data demonstrated the tumor suppressor role of SLAP in IHCC cells.

**Upregulation of SLAP suppresses Wnt activity and expression of downstream genes.** To determine the effects of SLAP on Wnt signaling, the β-catenin/TCF transcription reporter assay was conducted. Compared with Ad-NC expression (1.00±0.13 and 1.00±0.13), SLAP overexpression decreased the TOPflash activity (0.45±0.08 and 0.52±0.10), and no significant changes in the FOPflash activity were identified (P>0.01, P>0.001, t-test) (Fig. 3A). Furthermore, the expression levels of Wnt target genes, including β-catenin, c-Myc, Slug, Vimentin and MMP-9, were reduced in RBE and Huh28 cells overexpressing SLAP compared with Ad-NC (P<0.05 and P<0.01; Fig. 3B).

**Increased Wnt target genes in IHCC tissues.** The expression of Wnt target genes, including c-Myc, cyclin D1 and MMP-9, in IHCC tissues were also determined. As depicted in Fig. 4, the expression of Wnt target genes was significantly enhanced in IHCC tissues, compared with adjacent non-cancer tissues (P<0.01).

**SLAP overexpression decreases RBE and Huh28 cell invasion and migration.** Subsequently, the SLAP effects on IHCC cell invasion and migration were determined. Compared with Ad-NC expression, the migration and invasion capacities of cells were reduced following overexpression of SLAP in RBE (55.00±6.00) and Huh28 (76.00±5.00) cells compared with that of Ad-NC (162.00±12.00 and 135±8.00, respectively) (P<0.01 and P<0.001; Fig. 5), indicating the tumor suppressor role of SLAP in IHCC progression.

**Discussion**

In 2010, IHCC was reported as the second most common hepatic malignancy worldwide (1). It has been reported that the incidence of IHCC and the mortality rate of patients with IHCC continues to increase globally due to the lack of effective therapeutic strategies (20,21). To date, the molecular mechanisms underlying IHCC remain poorly understood; thus, the elucidation of the underlying mechanism may promote novel diagnostic and therapeutic approaches for IHCC treatment.

In the present study, the focus was primarily on SLAP, an adaptor protein. SLAP negatively controls RTK signaling through interactions with ubiquitin ligases, phosphatases, kinases and other signaling proteins (5). Abnormal SLAP expression has been identified in various cancer types (22), for instance, SLAP expression is reduced in acute myeloid leukemia (23). Previously, SLAP has been demonstrated to negatively regulate the wild-type c-Kit signaling in HL-60 cells, thereby modulating the progression of myeloid leukemia (7); however, the expression and role of SLAP in IHCC remains poorly understood.

To the best of our knowledge, the present study is the first to demonstrate that SLAP expression is decreased in IHCC tissues and cells, compared with controls. Further study indicated that SLAP overexpression suppressed IHCC cell proliferation and induced cell cycle arrest, demonstrating the tumor suppressor role of SLAP in IHCC progression. These data prompted an investigation into the underlying mechanism by which SLAP is involved in the progression of IHCC.

The abnormal activation of Wnt signaling has been frequently exhibited in patients with IHCC (24,25). The canonical Wnt signaling results in a transcriptional response with the transcription factor β-catenin as a key mediator (26). In normal cells, β-catenin is primarily located in the membrane and the cytoplasm with low expression patterns (26). The abnormal upregulation of β-catenin has been frequently observed in malignant cells, which is associated with enhanced cellular proliferation (2,27,28). In the present study, using the β-catenin/TCF transcription reporter assay, it was determined that SLAP overexpression suppressed Wnt signaling. Additionally, the downstream targets of Wnt signaling were also suppressed following SLAP overexpression. Consistent with the previous studies, it was demonstrated that SLAP overexpression induced IHCC cell migration and invasion.

To conclude, decreased SLAP expression may enhance IHCC malignant progression by activating Wnt signaling; however, further studies are necessary to elucidate the underlying potential mechanism by which SLAP inversely regulates Wnt/β-catenin activation.

**Acknowledgements**

Not applicable.

**Funding**

The present study was supported by the Doctoral Fund of Xiangya Hospital (grant no. XYH-20150312).

**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors’ contributions**

YWa performed the experiments and analyzed the data. XH, YWe, LL and WW performed a portion of the western blot experiments. NL designed the experiments, analyzed the data and gave final approval of the version to be published.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Xiangya Hospital, as stipulated by the Declaration of Helsinki, with written informed consent for the use of the specimens from all enrolled patients.

**Patient consent for publication**

Informed written consent for participation in the present study and use of the participant’s tissue was obtained from all participants and all patients consented to the publication of this study.
Competing interests

The authors declare that they have no competing interests.

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