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Original research article

Secretory leukocyte protease inhibitor (SLPI) as a diagnostic biomarker and facilitator of cell proliferation in cholangiocarcinoma

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ABSTRACT

Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor involved in inflammatory processes and has been implicated in various cancers. However, its role in cholangiocarcinoma (CCA) remains unclear. This study aimed to investigate SLPI expression in CCA tissues and serum, and to explore its effect on CCA cell proliferation. Immunohistochemical analysis of 50 CCA samples revealed significantly elevated SLPI expression compared to adjacent normal bile duct (NBD) and hepatocyte tissues ($p = 0.00136$). High SLPI expression was significantly associated with lymphatic metastasis ($p = 0.0312$), suggesting its relevance in tumor progression. Serum SLPI levels, measured by ELISA, showed a trend toward increased levels in CCA patients compared to healthy and *Opisthorchis viverrini* (OV)-infected individuals, with a statistically significant difference between CCA and hepatocellular carcinoma (HCC) patients ($p < 0.05$). Functional analysis using clonogenic assays demonstrated that treatment with recombinant human SLPI (rhSLPI) enhanced KKU-100 CCA cell survival in a dose-dependent manner, with 10 µg/mL rhSLPI significantly increasing the surviving fraction compared to the control group ($p = 0.0064$). These findings suggest that SLPI not only serves as a potential biomarker for distinguishing CCA from HCC but also contributes to CCA cell proliferation, highlighting its potential diagnostic and therapeutic relevance.

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1. Introduction

Cholangiocarcinoma (CCA) is a notably aggressive cancer arising from the epithelial lining of the bile ducts, representing around 15% of all primary liver malignancies globally.[1] The disease is particularly prevalent in Southeast Asia, with northeastern Thailand representing one of the highest incidence regions due to endemic infection with the liver fluke *Opisthorchis viverrine* (OV).[2,3] Chronic infection and sustained inflammatory responses in the bile duct epithelium play a key role in cholangiocarcinogenesis, leading to progressive epithelial transformation and tumor development.[4]

Despite ongoing efforts, early detection of CCA remains a major clinical challenge. The disease is often asymptomatic in its early stages and lacks specific serum biomarkers for routine screening. Moreover, intrahepatic CCA can be difficult to distinguish radiologically and histologically from hepatocellular carcinoma (HCC), particularly in patients with chronic liver disease, leading to misdiagnosis and suboptimal treatment strategies.[5,6] Thus, the identification of reliable and disease-specific biomarkers is critical for improving diagnostic accuracy and patient outcomes.

Secretory leukocyte protease inhibitor (SLPI) is a low molecular weight protein with known anti-inflammatory and antimicrobial functions. It inhibits serine proteases such as neutrophil elastase and plays a protective role in mucosal immunity.[7] Recent studies have implicated SLPI in tumorigenesis, showing that elevated SLPI expression contributes to enhanced cell proliferation, migration, and metastasis in various cancer types, including lung, ovarian, and colorectal cancers.[8,9] Conversely, SLPI has been shown to be downregulated in HCC, where it exerts tumor-suppressive effects by promoting endoplasmic reticulum stress and apoptosis.[10] These contrasting roles suggest that SLPI may have tumor-type-specific functions.

However, the role of SLPI in cholangiocarcinoma remains poorly understood. Given its divergent expression in different liver tumors, SLPI may serve as a potential diagnostic biomarker for differentiating CCA from HCC and could also play an active role in promoting CCA progression. Therefore, the present study aimed to investigate SLPI expression in CCA tissues and serum, and to determine its functional effect on CCA cell proliferation using in vitro models.

2. Materials and Methods

2.1 Tissue and serum samples

Formalin-fixed, paraffin-embedded tissue specimens including hepatocytes, normal bile duct (NBD), and cholangiocarcinoma (CCA) tissues (n = 50/group), as well as serum samples from healthy individuals (n = 9), *Opisthorchis viverrini* (OV)-infected individuals (n = 9), CCA patients (n = 7), and hepatocellular carcinoma (HCC) patients (n = 9), were obtained from Cholangiocarcinoma Research Institute (CARI), Khon Kaen University, Thailand. All samples were collected and processed following the institute's standard operating procedures (SOPs) for tissue preparation and serum collection. Written informed consent was obtained from all participants, and the study protocol received ethical approval from the Khon Kaen University Ethics Committee for Human Research following the Declaration of Helsinki (HE631037 and HE641574).

2.2 Cell line and culture conditions

The KKU-100 cell line, a poorly differentiated human cholangiocarcinoma cell line, was provided by the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). This cell was used for functional assays to assess the biological role of SLPI.

The cell was cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), enriched with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco). The cell culture was maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere.

2.3 Immunohistochemical (IHC) staining

Immunohistochemical analysis was investigated on paraffin-embedded tissue samples obtained from hepatocytes, normal bile duct (NBD), and cholangiocarcinoma (CCA) (n = 50). were deparaffinized, rehydrated, and washed through a series of xylene and ethanol baths, followed by distilled water. Antigen retrieval was performed using Tris-EDTA buffer (pH 9.0) in an autoclave at 121°C for 3 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 minutes and then blocked with 1% skimmed milk in phosphate-buffered saline (PBS) at room temperature for 1 hour. The tissue slides were then probed overnight at 4°C with a goat polyclonal anti-human SLPI primary antibody (1:50 dilution; AF1274, R&D Systems, Minneapolis, MN, USA). The slides were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG secondary antibody (1:100 dilution; P0449, Dako, Glostrup, Denmark) for 1 hour at room temperature. The signal was subsequently developed using 3,3'-diaminobenzidine (DAB) solution as the chromogen. Slides were counterstained with hematoxylin for 10 seconds. After dehydration and mounting, the slides were examined using light microscopy.

SLPI expression was assessed semi-quantitatively by applying the H-score method, which accounts for both staining intensity and the percentage of positively stained tumor cells. The H-score was calculated using the formula: H-score = [1 × (% cells with weak staining) + 2 × (% cells with moderate staining) + 3 × (% cells with strong staining)].[11]

The resulting scores range from 0 to 300. For each specimen, the entire tissue section was evaluated rather than selected microscopic fields. All evaluations were independently conducted by two blinded investigators, with discrepancies resolved by joint consensus.

2.4 Enzyme-linked immunosorbent assay (ELISA)

SLPI concentrations in serum samples were analyzed among four groups: healthy individuals (n = 9), individuals infected with *Opisthorchis viverrin*i (OV) (n = 9), cholangiocarcinoma (CCA) patients (n = 7), and hepatocellular carcinoma (HCC) patients (n = 9). For each sample, 100 µL of serum was used for the quantification of SLPI protein levels utilizing the SimpleStep ELISA® kit specific for SLPI (catalog no. ab263890, Abcam, Cambridge, UK), in accordance with the manufacturer's protocol.

Before analysis, all serum samples were diluted 1:100 using the provided NS sample diluent. The assay was conducted in duplicate for each sample to ensure reproducibility. Absorbance readings were taken at 450 nm, with background correction performed at 570 nm, using a microplate reader. SLPI concentrations were determined by interpolating from a standard curve generated from the known calibrators provided in the assay kit.

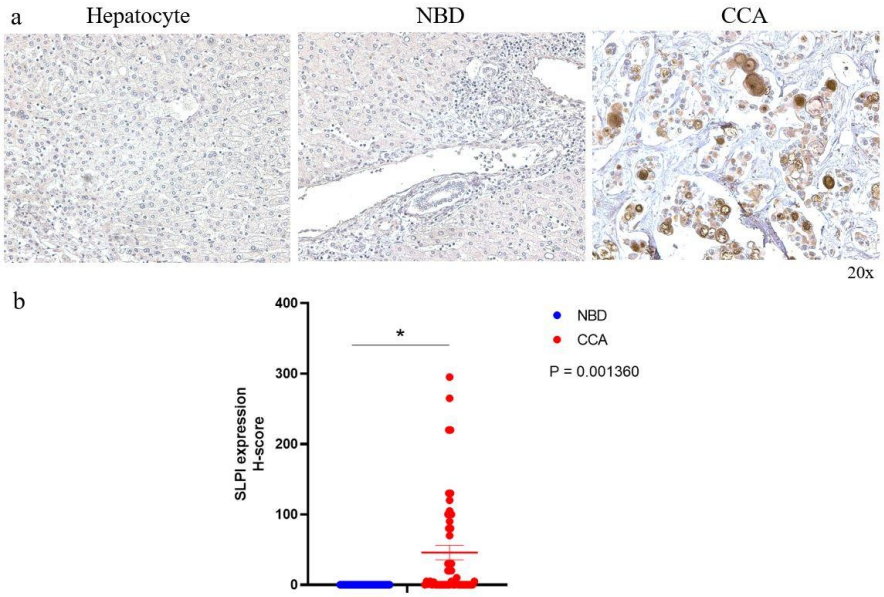

Fig. 1. Immunohistochemical analysis of SLPI expression in cholangiocarcinoma (CCA) tissues. (a) Representative images showing SLPI immunostaining in hepatocytes, normal bile duct (NBD), and CCA tissues. Images were captured at 200x total magnification (objective 20x). (b) H-score quantification of SLPI expression in NBD and CCA tissues (n = 50/group). Data are presented as mean ± SD. Statistical significance was determined using an unpaired Student t-test; *p < 0.05 (p = 0.00136).

2.5 Clonogenic assay

A clonogenic assay was employed to investigate the impact of recombinant human SLPI (rhSLPI; Sino Biological, China) on the viability of cholangiocarcinoma (CCA) cells. KKU-100 cell lines were seeded at a density of 1 × 10^2 cells per well in 24-well culture plates using RPMI-1640 medium enriched with 10% FBS. The cells were exposed daily to rhSLPI at concentrations of 0 µg/mL (control), 1 µg/mL, or 10 µg/mL for a period of 8 consecutive days, with both the medium and rhSLPI replenished each day to ensure consistent treatment.

Following the treatment course, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 minutes, and stained with 0.05% crystal violet for 15 minutes. Excess stain was removed by washing with PBS until the background became clear. Colony formation was assessed under a microscope, and the percentage of surviving colonies (%SF) was determined using the formula: %SF = (Number of colonies) / (Number of cells initially seeded × 100%).[12]

2.6 Statistical analysis

All data are expressed as mean ± standard deviation (SD) from at least three independent experiments, unless otherwise specified. Comparisons between two groups were performed using an unpaired Student's t-test, while differences among more than two groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For categorical variables, such as clinicopathological features, statistical comparisons were carried out using the chi-square test. A p-value less than 0.05 was considered statistically significant. All analyses and graphical presentations were performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA).

Table 1. Correlation between SLPI expression levels and clinicopathological parameters in CCA patients (n = 50). SLPI expression was classified as high and low according to IHC scoring. Statistical analysis was conducted using the chi-square test. *p-value < 0.05 indicates statistical significance.

	Patients (n=50)	SLPI expression(%)		x^2	p-values
		High	Low		
Sex				1.369	0.2419
Male	33	11(33.3)	22(66.7)		
Female	17	3(17.6)	14(82.4)		
Age(years)				0.1252	0.3539
≥64	27	7(25.9)	20(74.1)		
<64	23	7(30.4)	16(69.6)		
Lymphatic metastasis				4.641	*0.0312
Positive	17	8(47.1)	9(52.9)		
Negative	33	6(18.2)	27(81.8)		
Distal metastasis				2.624	0.1053
Positive	1	1(100)	0(0)		
Negative	49	13(26.5)	36(73.5)		

3. Results

3.1 SLPI expression in CCA tissue samples

To demonstrate the expression profile of secretory leukocyte protease inhibitor (SLPI) in cholangiocarcinoma (CCA), immunohistochemistry (IHC) analysis was performed on tissue slides from 50 CCA patients. The SLPI expression levels were compared with adjacent non-cancerous tissues, including hepatocyte and normal bile duct (NBD) regions.

As shown in Figure 1a, SLPI staining was markedly elevated in CCA tissues, with strong cytoplasmic staining observed in tumor cells, whereas minimal or no staining was detected in hepatocytes and NBD tissues. Quantitative H-score analysis confirmed a significant increase in SLPI expression in CCA tissues compared to NBD tissues (Fig. 1b). The mean H-score in the CCA group was significantly higher than in the NBD group ($p = 0.00136$), suggesting a tumor-specific upregulation of SLPI.

To determine the clinical relevance of SLPI expression, patients were stratified into high and low SLPI expression groups based on IHC scores, and correlations with clinicopathological features were analyzed (Table 1). While no significant associations were observed between SLPI expression and patient sex or age, high SLPI expression was significantly correlated with lymphatic metastasis ($\chi^2 = 4.641$, $p = 0.0312$). This finding suggests that SLPI overexpression may be associated with increased metastatic potential in CCA.

3.2 SLPI expression in serum samples

To address the potential of SLPI as a circulating biomarker, serum SLPI levels were quantified using enzyme-linked immunosorbent assay (ELISA) in four groups: healthy individuals (n = 9), individuals infected with *Opisthorchis viverrini* (OV; n = 9), patients with cholangiocarcinoma (CCA; n = 7), and patients with hepatocellular carcinoma (HCC; n = 9).As shown in Table 2, the mean with interquartile range (IQR) concentrations of SLPI were 24.94(23.47 - 32.72) ng/mL in healthy individuals, 30.00(28.14 - 41.58) ng/mL in OV-infected individuals, 44.06(19.75 - 60.28) ng/mL in CCA patients, and 23.61 (10.72 - 46.28) ng/mL in HCC patients.

Although no statistically significant differences were observed between the healthy, OV-infected, and CCA groups, there was a trend of increased SLPI levels in the CCA group. Importantly, SLPI levels in the serum of CCA patients were significantly higher than those in HCC patients ($p < 0.05$), suggesting the potential of SLPI as a differential biomarker between CCA and HCC (Fig. 2).

3.3 Effect of recombinant SLPI (rhSLPI) on CCA cell proliferation

To assess the functional role of SLPI in cholangiocarcinoma (CCA) cell proliferation, clonogenic assays were performed using KKU-100 cells treated with recombinant human SLPI (rhSLPI) at concentrations of 1 µg/mL and 10 µg/mL. As shown in (Fig. 3a), treatment with rhSLPI resulted in an increased number of visible colonies compared to the untreated control. Quantitative analysis of the surviving fraction (%SF) demonstrated a dose-dependent increase in colony formation. Specifically, the %SF values were $1.33 \times 10^{-4} \pm 5.77 \times 10^{-5}$ in the control, $4.67 \times 10^{-4} \pm 2.08 \times 10^{-4}$ in the 1 µg/mL rhSLPI group, and $8.67 \times 10^{-4} \pm 2.31 \times 10^{-4}$ in the 10 µg/mL group (Fig. 3b).

Statistical analysis revealed that treatment with 10 µg/mL rhSLPI significantly increased cell survival compared to control ($p = 0.0064$), indicating a proliferative effect of SLPI on CCA cells. Although treatment with 1 µg/mL rhSLPI showed an increasing trend, it did not reach statistical significance compared to control ($p = 0.1430$). These findings suggest that SLPI promotes CCA cell survival and proliferation in a dose-dependent manner.

Table 2. Comparative analysis of serum SLPI levels among study groups. Serum SLPI concentrations were measured by ELISA in healthy controls, OV-infected individuals, and patients with CCA and HCC. Data are presented as mean with interquartile range (IQR) along with the observed range (ng/mL) in each group.

Group	n	Mean(IQR)	Range(ng/mL)
Healthy	9	24.94(23.47 - 32.72)	16.2 - 37.3
OV infection	9	30.00(28.14 - 41.58)	23.9 - 67.5
CCA	7	44.06(19.75 - 60.28)	10.7 - 83.3
HCC	9	23.61(10.72 - 46.28)	0.2 - 53.6

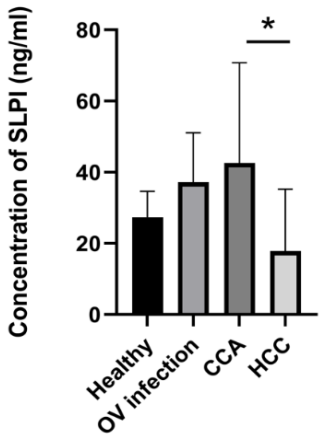

Fig. 2. Serum concentration of SLPI in healthy individuals, *Opisthorchis viverrini* (OV)-infected individuals, and patients with cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC). Data are presented as mean ± SD. Statistical significance was analyzed by one-way ANOVA followed by Tukey's post hoc test; *$p < 0.05$ vs HCC.

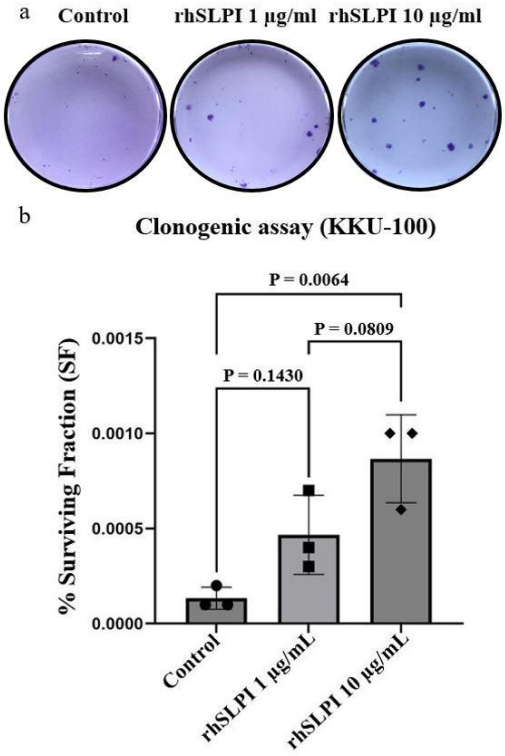

Fig. 3. Effect of recombinant SLPI (rhSLPI) on the clonogenic survival of cholangiocarcinoma cells (KKU-100). (a) Representative images of colony formation following treatment with 0 (control), 1 µg/mL, and 10 µg/mL rhSLPI. (b) Quantification of surviving fraction (%SF) from clonogenic assay. Data are presented as mean ± SD from three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; p-values are shown for the indicated comparisons.

4. Discussion

Secretory leukocyte protease inhibitor (SLPI) is a multifunctional protein that plays critical roles in modulating inflammation and innate immune responses by inhibiting serine proteases such as neutrophil elastase and cathepsin G13. While originally recognized for its anti-inflammatory and antimicrobial functions, recent evidence has implicated SLPI in various oncogenic processes, including cell proliferation, metastasis, and resistance to apoptosis.[16-21]

Cholangiocarcinoma (CCA) is a malignancy closely associated with chronic inflammation of the bile duct epithelium, often driven by infection with *Opisthorchis viverrini* (OV) in endemic regions such as northeastern Thailand.[14] In this study, we observed that SLPI expression was significantly elevated in CCA tissues compared to adjacent hepatocytes and normal bile duct (NBD) tissues. Notably, high SLPI expression was positively correlated with lymphatic metastasis, suggesting a potential role for SLPI in enhancing tumor aggressiveness in CCA. These findings support the notion that SLPI may serve as a specific tissue biomarker for CCA, distinct from hepatocellular carcinoma (HCC), in which SLPI expression is often downregulated.[10]

Interestingly, while SLPI overexpression was significantly associated with lymphatic

metastasis, no correlation was observed with distant metastasis. This discrepancy is most likely due to the very limited number of patients with distant metastasis in our cohort (n = 1), which precluded meaningful statistical analysis. From a biological perspective, SLPI may preferentially facilitate local invasion and lymphatic dissemination through extracellular matrix remodeling and cell motility, whereas the establishment of distant metastasis is a more complex process requiring additional molecular mechanisms. Larger patient cohorts will be required to clarify whether SLPI also contributes to distant metastatic spread.

Our serum analysis revealed a trend toward increased SLPI levels in CCA patients and a significant decrease in HCC patients, in line with previous reports in ovarian cancer showing SLPI elevation in malignancy.[15] Although the difference between CCA Although serum SLPI levels did not significantly differ between CCA and non-malignant groups, the observed difference between CCA and HCC suggests that SLPI may have potential as a differential indicator rather than a definitive diagnostic biomarker. Importantly, since normal individuals also exhibited higher serum SLPI levels compared to HCC patients, our current data cannot establish SLPI as a reliable diagnostic marker. Larger studies with expanded cohorts are warranted to further evaluate the diagnostic relevance of SLPI in distinguishing CCA from other liver diseases.

Functionally, we demonstrated that recombinant human SLPI (rhSLPI) enhanced clonogenic survival of CCA cells in vitro in a dose-dependent manner, indicating that SLPI contributes to cell proliferation and possibly tumor progression. This aligns with previous studies showing that SLPI promotes not only proliferation[17] but also invasion,[18] vasculogenic mimicry,[19] metastasis,[20] and anti-apoptotic signaling[21] in other cancer types. The exact molecular mechanisms by which SLPI facilitates these processes remain incompletely understood, but may involve modulation of NF-κB signaling, epithelial-to-mesenchymal transition (EMT), and matrix metalloproteinase (MMP) activity.[22-23] Although the clonogenic assay provided preliminary evidence that recombinant SLPI can enhance the growth capacity of CCA cells, it is important to note that this assay is primarily designed to evaluate cytotoxic effects rather than growth stimulation. The use of surviving fraction as a readout may therefore not fully reflect the proliferative effect of SLPI. Moreover, variation in colony size within treatment groups represents a limitation that reduces the clarity of interpretation. These findings should be further validated using dedicated proliferation assays such as cell counting, BrdU/EdU incorporation, Ki-67 immunostaining, or metabolic assays will be essential to confirm the pro-proliferative role of SLPI in CCA.

In this study, all CCA tissue specimens analyzed were derived from patients with OV-associated CCA, which represents the predominant form of cholangiocarcinoma in endemic areas of northeastern Thailand. Although our immunohistochemistry analysis focused on CCA tissues compared with adjacent hepatocytes and normal bile ducts, we did not specifically assess SLPI expression in non-malignant bile ducts from OV-infected individuals. Therefore, it remains unclear whether OV infection alone is sufficient to induce SLPI upregulation at the tissue level. Nevertheless, our serum analysis revealed a trend toward increased SLPI concentrations in OV-infected individuals compared with healthy controls, suggesting a possible link between OV infection and SLPI elevation. This observation is biologically plausible, as OV infection is known to trigger chronic inflammation of the biliary epithelium, which may contribute to the induction of SLPI expression. However, given the relatively small sample size in our cohort, these findings should be interpreted with caution. Larger studies including both OV-

infected non-malignant tissues and a greater number of serum samples are warranted to validate whether OV infection directly promotes SLPI expression and to better clarify the role of SLPI in the pathogenesis of OV-associated cholangiocarcinoma.

Given its distinct expression pattern in CCA and HCC, and its functional impact on CCA cell survival, SLPI represents a promising candidate for further investigation not only as a diagnostic biomarker but also as a potential therapeutic target with important pharmacological implications. Targeting SLPI-mediated signaling pathways could lead to novel therapeutic approaches to reduce tumor progression and metastasis in cholangiocarcinoma. In addition, detailed tumor staging information was not available in our dataset, which precluded further analysis of the relationship between SLPI expression and tumor stage. This represents another limitation of the study, and future investigations including comprehensive clinical parameters such as staging will be important to clarify whether SLPI expression correlates with tumor progression in cholangiocarcinoma.

5. Conclusion

This study demonstrates that SLPI is significantly upregulated in CCA tissues compared to adjacent normal bile duct and hepatocyte tissues. SLPI expression was positively associated with lymphatic metastasis, supporting its role in tumor aggressiveness. Although serum SLPI levels did not significantly differ between CCA and non-malignant groups, the distinction observed between CCA and HCC suggests a possible role for SLPI as a differential indicator. However, these findings should be interpreted with caution, and validation in larger clinical cohorts is necessary before any diagnostic application can be proposed.

Functionally, we demonstrated that recombinant SLPI promotes the proliferation of CCA cells in vitro. Although the concentrations used in clonogenic assays (1-10 μg/mL) were substantially higher than physiological serum levels and cannot be directly correlated with clinical conditions, these results provide supportive mechanistic evidence of SLPI's pro-tumorigenic role. Taken together, our findings highlight SLPI as a molecule of potential biological and clinical interest in CCA. Further studies with larger patient cohorts are warranted to confirm the diagnostic and therapeutic relevance of SLPI.

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Conflicts of Interest

The authors declare no conflict of interest.

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