RESEARCH ARTICLE

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Plasma S100A9 Levels in Healthy Individuals, *Opisthorchis viverrini*-Infected Individuals, and Patients with Cholangiocarcinoma

Piyaluk Danchaivijitr[1], Montinee Pholhelm[2,3], Sattrachai Prasopdee[2,3], Kritiya Butthongkomvong[4], Jutharat Kulsantiwong[5], Teva Phanaksri[3], Anthicha Kunjantarachot[3], Veerachai Thitapakorn[1,2]*

Abstract

Objectives: S100A9, a calcium-binding protein involved in inflammation and tumor progression, has been identified as a potential biomarker for *Opisthorchis viverrini* related cholangiocarcinoma (CCA) diagnosis. However, its role in *O. viverrini* infection remains unclear. **Methods:** ELISA was used to investigate the plasma S100A9 level among healthy individuals, *O. viverrini*-infected patients, and CCA patients to evaluate its potential as a diagnostic biomarker. The highest S100A9 levels were observed in CCA patients, while the lowest levels occurred in *O. viverrini*-infected individuals. **Results:** One-way ANOVA followed by post-hoc analysis revealed a significant difference between the *O. viverrini* infected and CCA groups, but not between the CCA and healthy groups or between the healthy and *O. viverrini* infected groups. S100A9 had high specificity (97.50%) but low sensitivity (21.88%) for distinguishing CCA from *O. viverrini* infected and healthy groups, suggesting limited utility as a standalone screening tool but potential value in diagnostic contexts. Although limited by diagnostic performance (AUC = 0.5580), the consistent upregulation trend in CCA aligns with previous studies. Combining S100A9 with other biomarkers may enhance diagnostic accuracy for *O. viverrini*-related CCA. **Conclusion:** S100A9 was elevated in CCA but not in *O. viverrini* infection, highlighting its potential as a differential diagnostic marker.

Keywords: S100A9- *Opisthorchis viverrini*- cholangiocarcinoma- Diagnosis

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Introduction

S100A9 was first reported as an antimicrobial protein released by neutrophils [1]. Since then, it has been extensively studied for its role in inflammation across both infectious and non-infectious conditions, leading to its identification as an inflammatory biomarker [2–7].

In terms of its inflammatory mechanism, S100A9 forms a heterodimer with S100A8, acting as an alarmin or as a component of the calprotectin complex and mediating pro-inflammatory responses [8–11]. In addition, S100A9 participates in signaling pathways associated with cancer-related processes [3, 12–14]. Cholangiocarcinoma (CCA) is a malignant tumor arising from bile duct epithelial cells and is associated with poor prognosis and high mortality due to asymptomatic early progression and late diagnosis [15]. Chronic inflammation plays a central role in cholangiocarcinogenesis by promoting free radical generation, DNA damage, and the accumulation of genetic mutations [16–19]. S100A9, a calcium-binding protein, is upregulated in CCA and has been proposed as a potential biomarker in liver fluke–endemic regions, where infection is a recognized risk factor [20–24], as well as in non-endemic areas in which primary sclerosing cholangitis predominates as a major risk factor [25,26]. In northeastern Thailand, the epidemiology of CCA is closely associated with chronic *O. viverrini* infection, which is classified as a Group 1 carcinogen [27].. Although S100A9 has been implicated in multiple parasitic infections, including leishmaniasis, filariasis, trypanosomiasis, and echinococcosis [8, 11, 28, 29], there is no clearly evidence from liver fluke–endemic regions such as Thailand, Vietnam, Laos, and Korea demonstrating that liver fluke infection promotes chronic inflammation through S100A9.

*[1]Dornsife College of Letters, Arts & Sciences, University of Southern California, Los Angeles, CA, USA. [2]Thammasat Research Unit in Opisthorchiasis, Cholangiocarcinoma, and Neglected Parasitic Diseases, Thammasat University, Pathum Thani, Thailand. [3]Chulabhorn International College of Medicine, Thammasat University, Pathum Thani, Thailand. [4]Medical Oncology Unit, Udonthani Cancer Hospital, Ministry of Public Health, Udon Thani, Thailand. [5]Faculty of Science, Udon Thani Rajabhat University, Udon Thani, Thailand. *For Correspondence: veebkk@gmail.com*

Interestingly, studies suggest that the upregulation of S100A9 primarily originates from infiltrating immune cells in the tumor stroma such as neutrophils and eosinophils, rather than from the CCA cells themselves [24, 28], similar to *Leishmani*a infection, where S100A9 is derived from infiltrating eosinophils [11]. Moreover, some reports suggest that S100A9 upregulation is not associated with clinicopathological features or disease progression in CCA [22].

However, among these candidates, S100A9 remains of particular interest because of its role in inflammation and tumor progression. In the absence of reports on S100A9 expression in *O. viverrini*- infected patients, a key question emerges as to whether elevated S100A9 levels are attributable to *O. viverrini* infection, cholangiocarcinoma, or a combination of both. Determining whether S100A9 expression is linked to *O. viverrini* infection, CCA, or both is essential for elucidating the transitional process in liver fluke-associated cholangiocarcinogenesis and for distinguishing CCA from *O. viverrini* infection. Therefore, this study aims to investigate and compare S100A9 levels among healthy individuals, patients infected with *O. viverrini*, and those with CCA.

Materials and Methods

Chemicals and reagents

S100A9 ELISA antibodies were purchased from Abcam Ltd. (Cambridge, UK). Maxisorp™ 96-well plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) was used for ELISA. BSA was purchased from AppiChem, Germany. The peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Sigma, Switzerland.

Subjects and sample preparation

The subjects were divided into three groups: 20 healthy individuals (HS), 20 *O. viverrini*- infected patients (OV), and 32 patients with cholangiocarcinoma (CCA). Healthy subjects were recruited based on normal physical examination findings and negative results from fecal examination for *O. viverrini* eggs. *O. viverrini* infection was confirmed by the presence of *O. viverrini* eggs by fecal examination. Diagnosis of CCA was confirmed by histopathological examination. The demographics of the subjects were shown in Table 1.

Enzyme linked immunosorbent assay (ELISA) of S100A9

The matched antibody pair kit S100A9 antibody (Cat. no. ab222271-5; Abcam, Cambridge, UK) was used according to the manufacturer's instructions. Briefly, all plasma samples were tested in duplicate. The capture antibody was diluted to 2 μg/mL in coating buffer (100 mM $NaHCO_3$, pH 8.5), and 50 μL of the diluted antibody was added to each well and incubated overnight at 4 °C. The plates were washed three times with 350 μL of washing buffer (10 mM phosphate-buffered saline, (PBS), pH 8.5, containing 0.05% Tween® 20), then blocked for non-specific binding with 300 μL of blocking buffer (1% bovine serum albumin in washing buffer) and incubated for 1 h at room temperature. The plates were washed with washing buffer, and the 50 μL of plasma (1:1 diluted in PBS) was added to each well and incubated at room temperature for 2 h on a shaker at 400 rpm. The plates were washed three times with 350 μL of washing buffer, followed by the addition of 50 μL of 0.5 μg/mL detection antibody diluted in blocking buffer. After incubation for 2 h at room temperature, the plates were washed three times with 350 μL of washing buffer. Subsequently, 100 μL of the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to each well and incubated for 20 min at room temperature in the dark. The reaction was stopped by adding 50 μL per well of 2 N sulfuric acid, and the absorbance was measured at 450 nm using a multifunctional microplate reader (VarioSkan, Thermo Fisher Scientific, Finland).

Statistical analysis

Statistical analyses were performed using SPSS Version 26.0 (Armonk, NY: IBM Corp.). To reduce the skewness of the ELISA absorbance at 450 nm, the data were log-transformed (natural logarithm), and the transformed values were used for dot plots and normality test. To identify statistically significant differences in plasma S100A9 levels among HS, OV, and CCA groups, a one-way analysis of variance (ANOVA) was conducted, followed by Scheffe's post hoc test.

GraphPad Prism version 10.5.0 (GraphPad Software, San Diego, CA, USA) was used to generate dot plots and to plot the area under curve of the receiver operating

Table 1. Summary of Subject Demography. All cholangiocarcinoma (CCA) subjects are stage 4 CCA.

	Group		
	Healthy	*O. viverrini*	CCA
Sample size	20	20	32
Sex (Male/Female)	5/15	11/9	18/14
Age (year)			
Min/Max	24/52	18/77	39/87
Mean ± SD	35.7 ± 7.5	50.5 ± 15.6	61.3 ± 12.1
Alcohol consumption			
No	8 (40%)	6 (30%)	10 (31%)
Yes	12 (60%)	14 (70%)	22 (69%)
Smoking			
No	18 (90%)	11 (55 %)	17 (53%)
Yes	2 (10%)	9 (45 %)	15 (47%)
Raw fish eating-habit (Source of *O. viverrini* infection)			
No	15 (75%)	4 (20%)	4 (12%)
Yes	4 (20%)	16 (80%)	28 (88%)
Uncertain	1 (5%)	0 (0%)	0 (0%)
History of *O. viverrini* infection			
No	19 (95%)	20 (100%)	26 (81%)
Yes	0 (0%)	0 (0%)	6 (19%)
Uncertain	1 (5%)	0 (0%)	0 (0%)
Fermented food eating-habit (Source of nitrosamine)			
No	1 (5%)	0 (0%)	0 (0%)
Yes	19 (95%)	20 (100%)	32 (100%)
Uncertain	0 (0%)	0 (0%)	0 (0%)

characteristic curve (AUC). The absorbance at 450 nm corresponding to the point of maximum likelihood ratio was used as the cut-off value for further analysis of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy, using the Diagnostic Test Evaluation Calculator (MedCalc Software Ltd., Ostend, Belgium; (https://www.medcalc.org/calc/diagnostic_test.php).

Results

Level of plasma S100A9 in healthy, O. viverrini-infected, and CCA subjects

Plasma S100A9 levels were measured by ELISA. The results showed that the highest level was observed in CCA subjects (mean = 0.2961, SD = 0.5518), followed by healthy group (mean = 0.1447, SD = 0.0967). The lowest level was found in *O. viverrini*-infected group (0.0612, SD = 0.0537) (Supplementary file 1). The range of S100A9 levels in CCA group showed a wider distribution compared to both healthy individuals and *O. viverrini*-infected subjects (Supplementary file 1). Dot plots of log-transformed absorbance values at 450 nm were generated for healthy, *O. viverrini*-infected, and CCA groups (Figure 1). One-Way ANOVA revealed a statistically significant difference in plasma S100A9 levels (P = 0.032), indicating differences between at least two groups. Scheffe's post hoc test indicated a significant difference between the *O. viverrini* infected and CCA groups (P = 0.049, 95% CI: -0.8535 to -0.0010). However, no significant difference was found between healthy individuals and the CCA group (P = 0.998, 95% CI: -0.4368 to 0.4157), or between healthy individuals and the *O. viverrini*- infected group (P = 0.095, 95% CI: -0.0561 to 0.8897) (Figure 1).

Diagnostic potential of S100A9

Analysis of the AUC revealed the following: between healthy and *O. viverrini*- infected subjects, the AUC was 0.7700 (95% CI: 0.6259–0.9141; cut-off < 0.02778) ; between healthy and CCA subjects, the AUC was 0.5641 (95% CI: 0.4092–0.7189; cut-off < 0.02925); and between *O. viverrini* infected and CCA subjects, the AUC was 0.6656 (95% CI: 0.5172–0.8140; cut-off > 0.1432) (Figure 2). To investigate the potential of S100A9 as a diagnostic biomarker for differentiating CCA subjects from healthy individuals and those with *O. viverrini* infection, the AUC was 0.5508 (95% CI: 0.4126–0.6890; cut-off > 0.3155) (Figure 2). Using 0.3155 as the cut-off, there were 7 true positives, 25 false negatives, 1 false positive, and 39 true negatives. The analysis yielded a sensitivity of 21.88% (95% CI: 9.28%–39.97%), specificity of 97.50% (95% CI: 86.84%–99.94%), positive predictive value of 87.50% (95% CI: 47.57%–98.18%), negative predictive value of 60.94% (95% CI: 56.33%–65.35%), and an overall accuracy of 63.89% (95% CI: 51.71%–74.88%).

Discussion

S100A9 plays roles in several biological processes, including inflammation, immune responses to infection [8–11], and carcinogenesis [3,12–14]. It has also been

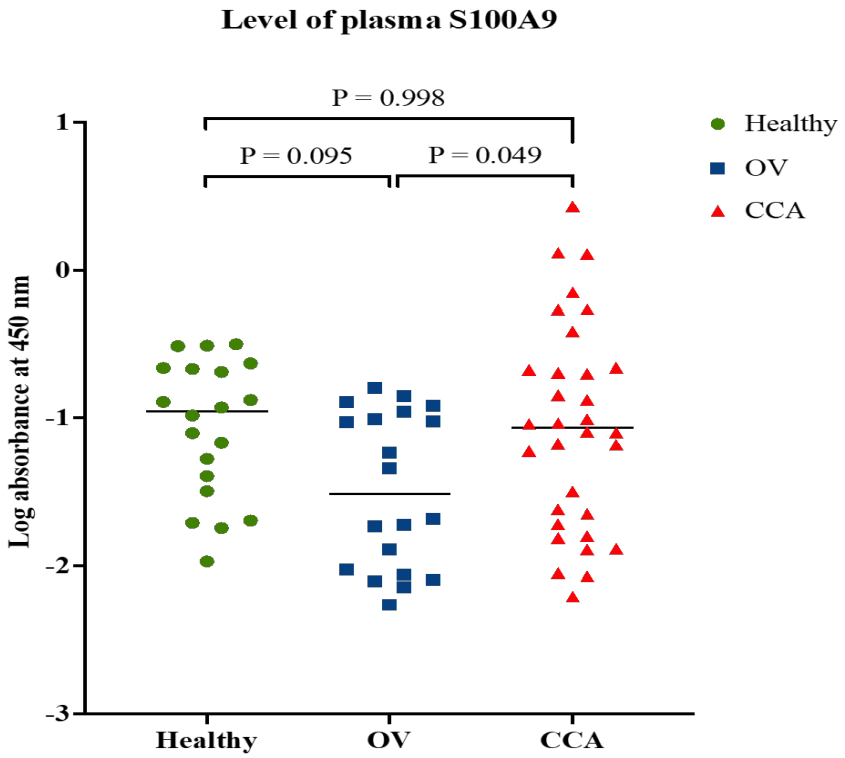

Figure 1 ELISA of Plasma S100A9 in Healthy (HS), *O. viverrini* infected (OV), and cholangiocarcinoma (CCA) groups. The green circle dot, blue square, and red triangle indicates the HS, OV, and CCA subjects, respectively. A significant difference was observed only between the OV and CCA groups (P=0.049), but not between the other groups.

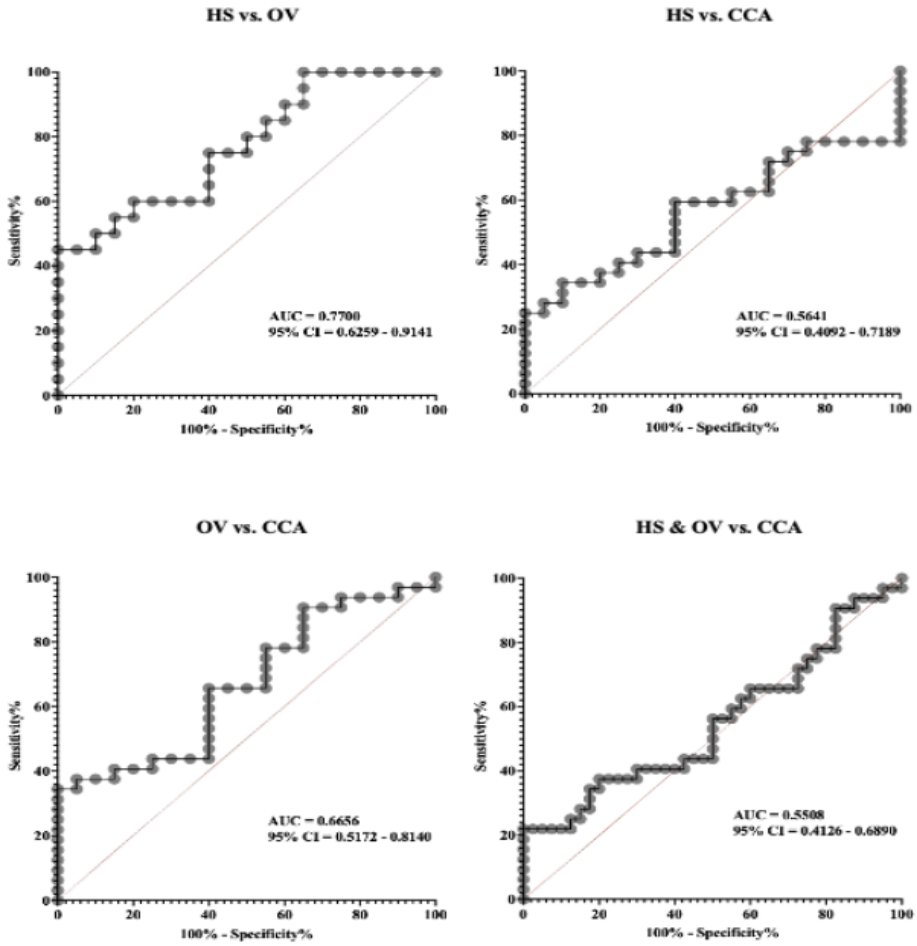

Figure 2. Area under Curve of the Receiver Operating Characteristic (ROC) Curve of Healthy (HS) and *O. viverrini* infected (OV) subjects, healthy (HS) and cholangiocarcinoma (CCA) subjects, *O. viverrini* (OV) infected and cholangiocarcinoma (CCA) subjects, and healthy (HS) & *O. viverrini* (OV) infected and cholangiocarcinoma (CCA) subjects.

identified as a key molecule involved in parasitic infections [8, 11, 28, 29] and as a potential diagnostic biomarker for various cancers, including cholangiocarcinoma (CCA) [20–26]. In *O. viverrin*-endemic regions, co-exposure to *O. viverrini* and nitrosamine compounds is considered a major risk factor for CCA [30]. However, the role of S100A9 in *O. viverrini* infection, a key precursor condition to CCA, remains unclear. Notably, S100A9 is primarily derived from infiltrating immune cells rather than from the CCA cells themselves [24, 28], which may contribute to inter-individual variability in immune responses and affect the degree of upregulation, thereby limiting its diagnostic utility.

In this study, S100A9 consistently exhibited an upregulation trend in CCA, consistent with previous reports. Although a statistically significant upregulation was observed between the *O. viverrini*- infected and CCA groups, no significant differences were found between the CCA and healthy groups or between the *O. viverrini* infected and healthy groups. Additionally, the AUC was not sufficiently high to support the use of S100A9 as a standalone diagnostic biomarker for CCA. S100A9 demonstrated high specificity (97.50%) but low sensitivity (21.88%), suggesting that it may be more suitable for differential diagnosis rather than for screening purposes. Several studies have recommended combining S100A9 with other biomarkers to improve overall diagnostic performance. Both tissue- and liquid-based biopsies have been employed to validate diagnostic biomarkers for CCA. Tissue-based immunohistochemistry identified S100A9 and CCTγ, which were co-identified by 2-D DIGE coupled with MALDI-TOF mass spectrometry and proposed for CCA diagnosis [25]. Moreover, combinations of S100A9 with AACT, NGAL, PSMA3, or with CA19-9, TRX, and CDHR2 showed high diagnostic performance based on plasma levels measured by sandwich ELISA (AUC > 0.9) [21, 22, 24, 25]. Nevertheless, the small sample size, low sensitivity, and modest AUC may limit its diagnostic power. Larger sample sizes, multicenter and combination with other biomarkers should be validated in future studies. The observed downregulation of plasma S100A9 in *O. viverrini* infected subjects, compared with both healthy controls and CCA patients, indicates that S100A9 is not elevated during *O. viverrini*- infection and supports its potential use as a biomarker for differentiating CCA from *O. viverrini* infection and healthy individuals. In this study, the plasma level of S100A9 is reported for the first time, helping to clarify the gap between *O.*

viverrini infection and CCA, particularly in regions where *O. viverrini* is endemic. This finding may contribute to improved diagnostic approaches for *O. viverrini*-associated CCA, especially in Southeast Asian countries. When the level of S100A9 is increased in the plasma of *O. viverrini*-infected patients or patients with a history of *O. viverrini* infection, awareness of CCA development should be raised.

In conclusion, two assumptions are proposed. First, S100A9 is upregulated during cholangiocarcinoma, supporting its potential as a biomarker to distinguish CCA from *O. viverrini* infection and healthy conditions. Alternatively, S100A9 may be suppressed during *O. viverrini* infection and may not play a significant role in the infection process. Overall, S100A9 demonstrates potential as a valuable diagnostic biomarker for identifying *O. viverrini*- related CCA.

Author Contribution Statement

Conceptualization: Thitapakorn V. Data Curation: Danchaivijitr P., Prasopdee S., Montinee Pholhelm, Kritiya Butthongkomvong, Kulsantiwong J., Phanaksri T., Kunjantarachot A., Thitapakorn V. Formal Analysis: Danchaivijitr P, Prasopdee S., Pholhelm M., Kritiya Butthongkomvong, Kulsantiwong J., Phanaksri T., Kunjantarachot A., Thitapakorn V. Funding Acquisition: Thitapakorn V., Prasopdee S. Investigation: Danchaivijitr P, Prasopdee S., Pholhelm M., Butthongkomvong K., Thitapakorn V. Methodology: Danchaivijitr P, Prasopdee S., Pholhelm M., Butthongkomvong K., Thitapakorn V. Validation: Danchaivijitr P, Prasopdee S., Thitapakorn V. Writing: Original Draft: Danchaivijitr P, Prasopdee S., Pholhelm M., Thitapakorn V. Writing, Review and Editing: Danchaivijitr P, Prasopdee S., Pholhelm M., Butthongkomvong K., Thitapakorn V.

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Ethical Declaration

This study was approved by the Human Ethics Committee of Udonthani Cancer Hospital, Udon Thani, Ministry of Public Health, Thailand, protocol number UCH-CT 11/2563.

Data availability

All data is available in this publication.

Declaration of generative AI in scientific writing

Only grammar correction and typographical edits were performed using the prompt "Typo and Grammar Check" by ChatGPT-4. No content generation, data analysis, or interpretation was conducted using AI.

Conflict of interest

The authors declared no competing interests for this study.

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