REVIEW

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Current State of Knowledge on Blood and Tissue-Based Biomarkers for *Opisthorchis viverrini*-induced Cholangiocarcinoma: A Review of Prognostic, Predictive, and Diagnostic Markers

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Abstract

Cholangiocarcinoma (CCA) is a prevalent cancer in Southeast Asia, with *Opisthorchis viverrini* (*O.viverrini*) infection being the primary risk factor. Most CCA cases in this region are diagnosed at advanced stages, leading to unfavorable prognoses. The development of stage-specific biomarkers for *Opisthorchis viverrini*-induced cholangiocarcinoma (Ov-CCA) holds crucial significance, as it facilitates early detection and timely administration of curative interventions, effectively mitigating the high morbidity and mortality rates associated with this disease in the Great Mekong region. Biomarkers are a promising approach for early detection, prognosis, and targeted treatment of CCA. Disease-specific biomarkers facilitate early detection and enable monitoring of therapy effectiveness, allowing for any necessary corrections. This review provides an overview of the potential *O. viverrini*-specific molecular biomarkers and important markers for diagnosing and monitoring Ov-CCA, discussing their prognostic, predictive, and diagnostic value. Despite the limited research in this domain, several potential biomarkers have been identified, encompassing both worm-induced and host-induced factors. This review offers a thorough examination of historical and contemporary progress in identifying biomarkers through multiomics techniques, along with their potential implications for early detection and treatment.

Keywords: Cholangiocarcinoma- *Opisthorchis viverrini*- opisthorchiasis- biological markers- prognosis

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Introduction

In the Greater Mekong subregion of Southeast Asia, *O. viverrini* causes live fluke-induced opisthorchiasis, which increases mortality rates associated with CCA [1]. In Thailand and other parts of Southeast Asia, CCA ranks as one of the most common cancers in males and females (96 per 100,000 men in Northeast Thailand) due to the high prevalence of liver fluke infection [2]. One main reason is having strong livelihoods and lifestyle associations within wetland ecosystems in the Mekong region, which are intricately related to human raw food consumption. The basin covers a large part of northeastern Thailand, almost the entire countries of Lao PDR and Cambodia [3, 4] (Figure 1A), where the custom of consuming raw fish exists (Figure 1B, D), which may have been contaminated with *O. viverrini* metacercaria [5, 6]. A fluke can settle in the small ducts of the intrahepatic bile ducts and live there for an average of 20–30 years (Figure 1C) [7]. Long-term *O. viverrini* infection causes bile duct inflammation, which leads to epithelial hyperplasia, periductal fibrosis, bile duct dilation, and, eventually, cancer [7].

According to the International Agency for Research on Cancer (IARC), opisthorchiasis, one of only three eukaryotic diseases classified as Group 1 carcinogens, can cause persistent inflammation in the biliary tract, causing cancer [8]. Chronic obstruction of bile ducts by flukes, mechanical damage caused by their suckers, the release of their excretory/secretory products, and immunopathology have all been noted as risk factors for the development of *O. viverrini*-associated CCA [9, 5, 10]. Intrahepatic cholangiocarcinoma (iCCA) is the most frequently diagnosed liver cancer subtype in places with a high prevalence of liver fluke infection, such as Northern Thailand. This is primarily due to a prototypical inflammatory etiology of liver fluke disease (Figure 1D) [11, 12]. Flukes normally reside in the small to medium intrahepatic bile ducts (IHCC), but in severe infections, they may also settle in the gallbladder and extrahepatic ducts (EHCC), which ultimately raises the risk of extrahepatic cholangiocarcinoma (eCCA) [13].

It is often difficult to treat patients with CCA, whether

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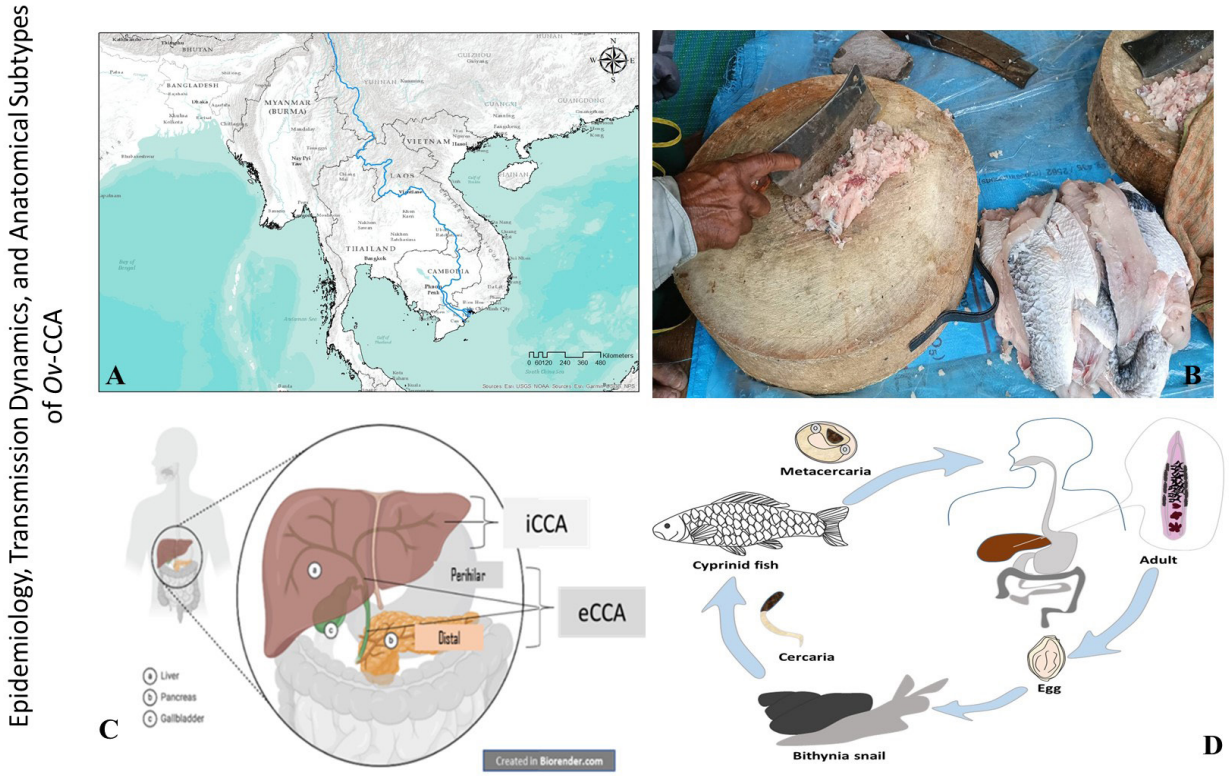

Figure 1. Epidemiology, Transmission Dynamics, and Anatomical Subtypes of Ov-CCA. A. An illustration of the Lower Mekong Basin in Southeast Asia showing where the highest prevalence of CCA is found in Thailand, Lao PDR, Cambodia and Vietnam. B. Raw or fermented fish consumption is the main cause of *O. viverrini* infection (freshwater cyprinid fish). A diet high in raw fish causes individuals to experience cycles of O. viverrini infection, treatment, and re-infection, increasing the risk of developing CCA in highly endemic areas. C. Schematic diagram of CCA types: Based on anatomical structures, CCA is divided into two subtypes: extrahepatic (eCCA) and intrahepatic (iCCA). Extrahepatic CCA can be further classified into perihilar (pCCA), middle, and distal (dCCA), depending on the locations of the tumor. D. Life cycle of liver fluke in humans: The human bile duct is a host environment for the parasite *O. viverrini* sexual development. After a brief time of free-living growth, eggs produced by the cross-fertilization of hermaphroditic adult liver fluke move through feces and infect the first intermediate host (snails). The reproduction in this host environment is asexual. Infected snails (Bythinia Spp.) release larval flukes (cercariae) that then develop into encysted metacercariae in a second intermediate host of specific fish species. (Cyprinid spp.). Ingestion of fish infected with metacercariae leads to the infection of the human host.

Opisthorchis viverrini-associated cholangiocarcinoma (Ov-CCA) or non-*Opisthorchis viverrini*-associated cholangiocarcinoma (non-Ov-CCA), due to the lack of early signs and biomarkers [14]. Most people with Ov-CCA suffer from advanced metastatic disease when detected, resulting in poor prognosis and short survival time [15]. The 5-year survival rate is still quite low despite advancements in diagnostic techniques such as blood tumor markers, radiographic and endoscopic imaging, and pathological analysis of biopsies or endoscopic brushings [16-18]. Hence, to address ascending rates of CCA cases in the endemic region by late diagnosis due to the lack of early symptoms and the refractory nature of these tumors, improved biomarkers and diagnostic methods are needed for detection in humans and susceptible animal host to prevent progression to cancer (CCA) from controlling and monitoring *O. viverrini* effectively.

Researchers continue using conventional serum markers despite advancements and discovering prospective biomarkers. They have utilized carbohydrate antigen 19-9 (CA 19-9) and carcinoembryonic antigen (CEA) for routine CCA screening. However, these markers are also elevated by alcoholic liver disease, chronic viral hepatitis, primary sclerosing cholangitis (PSC), cholestasis, liver damage, and various malignancies [19, 20]. Currently, there are no tumour-specific markers for Ov-CCA. Hence, until now, ultrasound has been the only method convincingly used to screen for CCA due to *O. viverrini* [21, 22]. However, pathognomonic radiological features in imaging frequently make accurate diagnosis difficult, leaving biopsy as the only option for determining CCA [23]. Thus, a strategic road map for neglected tropical diseases 2021-2030 establishes global targets for preventing, controlling, eliminating, and eradicating a diverse set of NTDs, including the *O. viverrini* parasite, and effectively necessitates diagnostic biomarkers for early infection, recurrence, or documentation of successful treatment.

Even though there are many examples of lab-based experiments and small-scale human trials showing the usefulness of new biomarkers in the field of Ov-CCA [24-26], finding biomarkers and figuring out how to use them

in clinical settings remains a major challenge. Identifying biomarkers that differentiate disease phenotypes, such as symptomatic versus asymptomatic or uncomplicated versus severe forms, holds substantial promise for improving prognostication and providing precise guidance for clinical researchers, physicians, and surgeons. This review aims to find potential biomarkers for clinical use in diagnosing and monitoring *O.viverrini* infection. Hence, this review serves as an up-to-date overview of identifying and validating potential biomarkers for Ov-CCA. Additionally, it discusses potential markers and biomarker studies involving Ov-CCA, investigates the reasons behind limited implementation in clinical settings, and proposes pilot studies, machine learning, and "omics" technologies for future biomarker research and clinical translation.

Biomarker

A biomarker refers to any characteristic or measurement of a biological sample or medical condition that can be accurately and reliably measured. In other words, it is a way to describe or quantify features of a biological system [27, 28]. On the other hand, the term "marker" is more encompassing, referring to any discernible attribute, substance, or indicator that can be employed to identify, measure, or monitor a particular condition, disease, or biological process [29]. For a long time, researchers and healthcare professionals have hailed biomarkers as the key to improving patient care and reducing medical expenses [30]. They are beneficial in detecting and grading disease seriousness in laboratory and clinical settings. The impact of biomarkers on cancer therapy can be assessed using a three-faceted framework that includes prognostic, predictive, and diagnostic assessment [31] (Table 1). We will highlight these three areas of biomarker investigations in Ov-CCA for this review and their relevance to cancer.

Cancer researchers categorize cancer biomarkers into three groups based on their associated signature: prognostic, predictive, and diagnostic. Regardless of any medical intervention, prognostic biomarkers provide information about the tumor's severity and the patient's long-term prognosis. Predictive biomarkers, on the other hand, provide information about how a therapeutic intervention will affect a patient. Finally, diagnostic biomarkers are biological criteria that help diagnose a disease and may indicate its progression or therapy efficacy.

Biomarkers for Opisthorchis viverrini (Ov) infection

Currently, the "gold standard" detection method for diagnosing opisthorchiasis is detecting *O. viverrini* eggs in stool samples using the formalin ethyl-acetate concentration technique (FECT). This approach exhibits a restricted diagnostic sensitivity and specificity for detecting mild infections, as eggs of *O. viverrini* parasites are often indistinguishable from those of minute intestinal flukes (MIFs) when present in fecal matter [32, 33]. Detecting chronic *O. viverrini* infection through coprological methods presents a significant challenge due to the obstruction of egg flow into the feces caused by opisthorchiasis. Further complicating identification is fibrosis or primary biliary sclerosis in the bile ducts. As a result, conventional diagnostic approaches, such as microscopic examination of stool samples, may not be effective in identifying the presence of *O. viverrini* infection in individuals with chronic opisthorchiasis [34, 35]. In addition, numerous investigations have shown that the relationship between estimated CCA incidence and fecal Ov eggs count is only weakly associated with active infection during CCA [36, 37]. Doctors and patients in endemic opisthorchiasis areas are currently facing a significant challenge. In order to aid in the early detection, prognostication, and therapy of this form of cancer, it is imperative to look into the molecular mechanism behind the development of CCA.

High-throughput technologies have paved the way for identifying molecular markers of disease processes by comprehensively analyzing genes, transcripts, proteins, and other biological molecules [38]. Given the multitude of biomarkers and their diverse functions and properties, selecting and designing clinical trials involving biomarkers can be challenging. Therefore, this review addressed potential biomarkers for Ov-CCA, which could provide researchers and clinicians with a reliable panel of biomarkers. Importantly, these biomarkers may also have the potential to identify CCA not attributed to *O. viverrini,* as CCA can arise as a consequence of either condition.

Biofluid (serum and plasma) and tissue biomarkers

Serum and plasma are used to identify potential biomarkers because of their ease of collection, stability, and wide range of biologically active molecules, which can provide valuable information about a person's health status. The choice between serum and plasma ultimately depends on the specific biomarkers of interest and the intended downstream applications. The most effective biomarkers were those demonstrating the highest levels of both sensitivity and specificity. Other popular methods for assessing the quality of biomarker signatures include measuring the area under the curve and determining receiver operating characteristics (ROC) (AUROC) [39]. Human serum may include antigens from the circulating

Table 1. Biological Markers Types and Examples in Clinical Application

Marker Type	Definition	Examples in Clinical application
Diagnostic marker	A biological marker is used to diagnose a specific disease or condition.	PSA (prostate-specific antigen) test for prostate cancer (Ilic et al., 2018)
Predictive marker	A biological marker helps predict how a disease will develop or how a patient will react to a certain treatment.	HER2 (human epidermal growth factor receptor 2) gene for breast cancer treatment (Zhang et al., 2020)
Prognostic marker	A biological marker helps predict the future outcome of a disease, such as the likelihood of survival or progression.	LDH (lactate dehydrogenase) levels for Hodgkin's lymphoma prognosis(Qi et al., 2021)

liver fluke that can be utilized to diagnose liver fluke infection. Based on the information in (Table 2), the review will focus on blood and tissue-based biomarkers that exhibit high sensitivity, specificity, and accuracy in detecting *O. viverrini* infection and Ov-CCA in human or hamster models.

CA19-9 and CEA are widely used prognostic biomarkers [40, 41] in CCA, due to their proven clinical utility [42]. CA19-9 has a sensitivity of 50-90% and a specificity of 54-98% [43- 46], while increased bile CEA levels predict CCA with a sensitivity of 58-84% and specificity of 33-84% [47, 46]. Various alternative biomarkers have been suggested for Ov-CCA, potentially providing higher efficacy and specificity. However, their clinical utility and reliability necessitate rigorous research and validation studies. Despite this, the use of these alternative biomarkers has shown promising results, and they may offer improved predictive, prognostic and diagnostic value for Ov-CCA in the future.

Heat shock proteins (HSPs)

Heat shock proteins (HSPs) are proteins produced by cells in response to stress and identified as a therapeutic target for various cancers [48- 52]. Furthermore, evidence linked HSP overexpression to tumor aggressiveness, metastasis, and poor prognosis [53, 54]. Under stressful circumstances, HSPs either maintain the stability of particular proteins or cause their proteasomal breakdown, assisting in cell survival [55].

A heat shock proteins (HSPs) isoform, HSP70, a conserved protein in mammalian species, shows a sensitivity of 90% and specificity of 100% for CCA detection [56]. A higher sensitivity and specificity than CA19-9 and CEA makes it a potentially better biomarker for CCA detection. *O. viverrini* infection causes oxidative stress, which in turn causes DNA damage, aberrant tissue remodeling, and changes in gene expression, all of which have been linked to the development of cancer [57]. A unique isoform of HSPs, Hsp90, destroys associated oncoproteins and reduces the growth rate of a wide range of cancers [58].

In *O. viverrini* infection, particularly HSP90α antibodies may have the potential as a diagnostic biomarker for cholangiocarcinoma. The anti-HSP90α serum levels had a sensitivity of 76.2% and specificity of 71.4% in discriminating cholangiocarcinoma from healthy individuals, as well as other diseases like cirrhosis and hepatitis, but not colon cancer, and their levels correlated with tissue expression [59]. Rucksaken and colleagues conducted a study in 2014, revealing that individuals with Ov-CCA had more autoantibodies targeting HSP70, RNH1, and ENO1. Healthy people, on the other hand, had lower levels of these autoantibodies. Furthermore, combining positivity rates for HSP70, ENO1, or RNH1 autoantibodies increased the specificity of detection to more than 78% [60]. As a result, serum anti-HSP90 levels and other markers may have significant clinical value for detecting Ov-CCA early and predicting the disease stage.

Phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K)

Cancer cells metabolism, motility, proliferation, and survival are controlled by a key oncogenic pathway known as phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) [61, 62]. The potential and suitability of phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PIK3CB), a specific isoform of the catalytic subunit of PI3K, as biomarkers were assessed using indirect ELISA for the diagnosis of *O. viverrini* infections and CCA. In a study conducted by Prasopdee, Yingchutrakul et al. (2022), the potential and suitability of phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform (PIK3CB) as a biomarker was assessed using indirect ELISA for the diagnosis of *O. viverrini* infections and CCA. The plasma levels of PIK3CB were significantly different between the *O. viverrini* and CCA groups compared to the non-Ov CCA group. However, there was no significant difference in PIK3CB levels between the Ov and CCA groups. The sensitivity and specificity values for detecting *O. viverrini* using an OD450 cut-off at >1.570 were 76% and 72%, respectively [63]. Previous research has acknowledged that the downstream signaling molecules, specifically the PI3K signaling pathways involving AKT and ERK, are crucial in stimulating cholangiocyte proliferation [64]. In Ov-CCA investigations, the PI3K/AKT/mTOR and ERK pathways were mainly reported in tissues and cell lines [65, 66]. Inhibitors of PI3K have significant potential for treating CCA and are important as a cancer diagnostic [67]. To test for Ov-CCA and non-Ov-CCA, the measurement of plasma PIK3CB via indirect ELISA holds great potential.

Interleukin-6 (IL-6)

Chronic inflammation is a crucial factor in the development of O. viverrini-induced CCA. In advanced cases of chronic *O. viverrini* infection, a high level of circulating plasma IL-6, a well-known inflammatory cytokine, is related to the severity of periductal fibrosis but not to the infection itself [68]. During *O. viverrini* infection, injured epithelial cell lining can produce several cytokines, including IL-6, IL-8, TGF- β and TNF- α, leading to chronic bile duct inflammation [69], causing bile duct epithelial cell proliferation and impaired epithelial barrier function.

IL-6 plasma levels (>64 pg/mL) showed 80% sensitivity and 90% specificity for detecting CCA with an 89% accuracy (95% CI = 85% to 93% [68]. However, the low diagnostic specificity of IL-6 is worth noting, as it has been proposed as an inflammatory marker for many conditions, including other cancers [70- 72], virus [73] and bacterial infections [74, 75].

The usefulness of plasma IL-6 as a diagnostic marker for cholangiocarcinoma (CCA) is limited due to its nonspecific rise in response to immune challenges other than parasite antigens. However, given the high prevalence of exposure to the carcinogen *O. viverrini* through the consumption of raw fish in half the high-incidence population, a readily available biomarker like plasma IL-6 could have significant clinical utility in identifying individuals at risk for CCA or those with early-stage disease [76]. In the context of Isaan, Thailand, where the prevalence of *O. viverrini* infection can reach as high as

Table 2. Blood and Tissue based Protein and Antibody Biomarkers in OV-CCA

Biomarkers	Study based	Potential clinical Use			Detection techniques	"Expression /localization"	Test accuracy indices			Ref. author
Only markers are labelled as M		Diag.	Prog.	Pred.			Sensitivity	Specificity	Accuracy	
					Serum/Plasma/ Tissues					
Rhophilin associated tail protein1- like (OvROPN1L) GenBank: KJ7 19301	Humans (Ov infected) and hamsters	√			ELISA (Synthetic peptide P1) peptide P1 absorbance value *O. viverrini*- infected sera (P<0.0001)	High antigenicity (based on absorb-ance) serum	Low (No quantit ative value mentio ned)	High (No quantitative value mentioned)	-	(Geadkaew-Krenc et al., 2020)
Anti-Heat Shock Protein 90α,	Humans and hamsters	√	√		ELISA	High antigenicity Serum	76.2%	71.4%	75.5 %	(Rucksaken et al., 2014; Boon-jaras pinyo et al., 2015)
Platelet-Derived Growth Factor Alpha (PDGFA)	Humans and hamsters	√	√		qPCR, IHC	Higher Serum	-	-	-	(Boonjaras pinyoet al., 2012)
CCA- associated carbohydrate antigen (CCA- CA) (S121 (mAb) against glycan epitope on MUC5AC) M	Humans		√	√	Immunohistochemistry	Higher Serum Tissue	87.6%	89.6%	-	(Sawanyawisuth et al., 2012) (Wongkham et al., 2003)
Checkpoint kinase1 (Chk1)	Humans	√			Indirect ELISA	Higher Plasma	59.38 %	85.71 %	71.67 %	(Phanaksri et al., 2022a)
PTP alpha and fibronectin	Humans and hamsters	√			LC-MS/MS, WB and IHC	Higher Plasma	-	-	-	(Khoontawad et al., 2012)
Phosphatidylinositol 4,5- bisphosphate 3-kinase catalytic subunit beta isoform (PIK3CB)	Humans	√	√		(LC-MS/MS) indirect ELISA	Higher Plasma	76%	72%		(Prasopdee et al., 2022b)
YKL- 40/chitinase- 3-like protein 1	Humans		√		ELISA IHC	Higher Serum Tissues	-	-		(Thongsom et al., 2016)
S100 calcium binding protein	Humans	√	√		ELISA	Higher Serum Tissue	71.4%	71.7%		(Wu et al., 2016)
Plasma hydroxyproline, Collagen I, MMP-7 and HYP	Humans	√		√	ELISA and zymography	Higher plasma	Collagen 73.1% MMP-75.8 % HYP 78.5%	77.6 % 72.5 % 74.9 %		(Prakobwong et al., 2012)
Polymeric immunoglobulin receptor (PIGR)	Humans	√		√	LC-MS/MS and sandwich ELISA	Higher Plasma	78%	71%	73%	(Prasopdee et al., 2022a)
IL-6 (Intereukin-6)	Humans and hamsters			√	ELISA	Plasma	80 %	90%	89%	(Sripa et al., 2012)
oxidized alpha-1 antitrypsin (ox-A1AT) M	Humans			√	ELISA	serum	100% healthy vs. *O. viverrini*- infection (Predic tive)	90% healthy vs. *O. viverrini*- infecti on (Predic tive)	-	(Jamnongkan et al., 2013)
Orm2 and KIF18A	Hamsters	√			LC-MS/MS, WB, IHC	Plasma and Tissue	-	-	-	(Rucksaken et al., 2012)
Exostosin 1	Hamsters			√	PAGE/LCMS- MS	Plasma and Tissue	91.7%	50%	-	(Khoontawad et al., 2014)
IgG antibodies for Ov-positive CCA M	Humans	√			ELISA	Serum and tissues	99.2%	93 %	-	(Titapun et al., 2020) (Tesana et al., 2007)
14-3-3 eta (risk assessment marker in endemic areas)	Humans and Hamsters	√			Tissue microarrays (TMAs) and Im-munohistochemistry (IHC)	Tissues	-	-	-	(Haononet al., 2016)
Annexin (ANXA1 and 2)	Humans and Hamsters		√		Tissue Microarray (TMA) and Im-munohistoc hemistry (IHC)	Tissues and Cell lines	-	-	-	(Yonglitthipagon et al., 2010; Hongsrichan et al., 2013)
Opisthorchis viverrini granulin 1 (Ov-GRN-1)	Hamsters		√		Immunohistochemistry,immunoass ay, RT-PCR	Tissues	-	-	-	(Upontain et al., 2018)
Plasma checkpoint protein 1 (Chk1)	Humans		√		LC-MS/MS ELISA	Plasma	59.38 %	85.71 %	-	(Phanaksri et al., 2022b)

Abbreviations:, PIK3CB, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta; Chk1, Checkpoint kinase 1; YKL-40, Chitinase-3-like protein 1; CSC markers, Cancer stem cell markers; PDGFA, Platelet-derived growth factor subunit A; KLK11, Kallikrein-11; CD44v9, CD44 variant 9; IL-6, Interleukin 6; Anti-Hsp90α, Antibodies against Heat shock protein 90 kDa alpha; ANXA1 and ANXA2, Annexin A1 and Annexin A2; ox-A1AT, Oxidized alpha-1-antitrypsin; Exostosin 1, Exostosin glycosyltransferase 1; Rhophilin associated tail protein, Rhophilin associated tail protein 1 Plasma hydroxyproline, Collagen I, MMP-7, and HYP: Plasma hydroxyproline, Collagen type I, Matrix metallopeptidase 7, Hydroxyproline; Anti-Hsp, Antibodies against Heat shock proteins; IgG antibody for (Ov + CCA), Immunoglobulin G antibody against *Opisthorchis viverrini and* cholangiocarcinoma; 14-3-3 eta, 14-3-3 protein eta 1 Orm2; KIF18A, Orosomucoid 2 and Kinesin family member 18A PTP alpha; fibronectin, Protein tyrosine phosphatase alpha and fibronectin; PIGR, Polymeric immunoglobulin receptor; TLS, Translocated in liposarcoma; VSX2, Visual system homeobox 2 beta; S100, S100 calcium-binding protein; MUC5AC, Mucin-5AC

79%, there exists an urgent need for an easily accessible immune marker capable of discriminating between the infection and the subsequently advanced pathology it induces, especially given the region's disproportionately high incidence of intrahepatic cholangiocarcinoma [77].

CCA-associated carbohydrate antigen (CCA-CA)

Monoclonal antibodies (mAbs) are required to effectively target cancer cell-specific antigens while reducing binding to normal cells. As a result, they are useful biological tools in immunodiagnostic procedures [78]. When using the mAb method to investigate novel markers in various cancers, high sensitivity and specificity have been achieved [79].

S121 monoclonal antibody (mAb) recognizes an unidentified glycan epitope on MUC5AC, referred to be CCA-associated carbohydrate antigen, in Ov-CCA [80]. The antigen was found to be a glycan epitope and shown to be reactive to an S121 immunoglobulin M MoAb [80]. MUC5AC is an O-glycosylated glycoprotein that is part of the membrane-bound and secreted epithelial mucin family. It has the most potential to be a predictive biomarker [81].

In a hamster model, CCA-CA expression increased gradually with tumor progression from Ov-CCA, making it an excellent time-dependent CCA-CA marker [82]. An enzyme-linked immunosorbent assay (ELISA) utilizing lectin-captured MUC5AC was developed which can differentiate patients with cholangiocarcinoma (CCA) from healthy controls, individuals with active *O. viverrini* infection, and patients with various gastrointestinal malignancies, hepatocellular carcinoma, and benign hepatobiliary diseases, with high sensitivity (87.63%) and specificity (89.58%) [82].

Furthermore, elevated serum CCA-CA levels correlated with poor patient outcomes [83, 80]. A meta-analysis has indicated that the detection of mucin 5AC (MUC5AC) in serum samples may serve as a potent biomarker for CCA, offering a specificity of up to 97% and sensitivity of 63% [80, 84, 85] . Also, most biliary tract cancer (BTC) tumor biopsies from patients have shown high MUC5AC reactivity. This suggests that the MUC5AC antigen associated with the tumor is released into the bloodstream, where it can be found [86]. Hence CCA-CA has the potential to be a new marker for early-stage CCA, and a panel of two markers, CA19-9 and MUC5AC, could effectively distinguish CCA from non-CCA with 70% sensitivity, 82.5% specificity, and AUC 0.806 [87]. These findings have important implications for improving disease diagnosis and management strategies

S100 calcium-binding protein

The S100 protein family is made up of a variety of small acidic calcium proteins, each with its own set of functions [88]. S100 calcium-binding protein A9 (S100A9), a putative pro-inflammatory mediator in both acute and chronic inflammatory processes, plays a significant role in the pathogenesis of inflammation-associated carcinogenesis [89, 90]. Duangkumpha, et al. (2019) study found significantly higher concentrations of S100A9 protein in the sera of CCA patients than in normal control groups. According to their published results, S100A9 was a promising diagnostic biomarker with sensitivity, specificity, and an AUC value of 0.888, equal to the differential diagnosis of CCA and normal control [91].

A recent study by Kimawaha and colleagues (2021), the diagnostic accuracy of CCA patients with low CA19-9 levels can be improved by using S100A9 as a complementary marker. S100A9 has a high diagnostic yield of 95% in this patient population. Combining S100A9 with CA19-9 further enhances the diagnostic efficiency, increasing the sensitivity value from 78% for S100A9 alone to 95% for the two markers together. Decision Tree analysis helped identify this complementary relationship between the two biomarkers [87]. Many benign biliary disorders (BBD) have a markedly elevated serum level of S100A9 [92].

In a study on Ov-CCA tumor tissue, Wu and colleagues (2016) found that the expression of the calcium-binding protein S100P was significantly higher in Ov-CCA and correlated with poor patient survival. Cox regression analysis revealed that high S100P expression was an independent prognostic factor for overall survival. Moreover, the authors measured CCA patients' S100P levels in serum and bile fluid samples. They found that S100P levels were higher in these patients than in healthy individuals or *O. viverrini*-infected patients. S100P expression was significantly associated with advanced tumor stage, metastasis, and poor patient survival. Furthermore, knockdown of S100P expression suppressed cholangiocarcinoma proliferation, caused cell cycle arrest, promoted apoptosis, and augmented the sensitivity of cholangiocarcinoma cells to sunitinib and apigenin [93].

Despite suggestions of the S100P protein as a potential novel prognostic biomarker of colorectal cancer [94, 95]. A meta-analysis demonstrated that the prognostic value of S100P significantly correlates with reduced overall survival in patients with cholangiocarcinoma and hepatocellular carcinoma, but not in patients with gastric, colorectal, gallbladder, or pancreatic malignancies [96]. Further research is needed to explore the potential diagnostic usefulness of S100A9 in combination with CA 19-9, especially in cases where the CA19-9 level is normal or low. The goal is to identify a blood-based biomarker panel that can aid in diagnosing and predicting outcomes for Ov-CCA. Studies have demonstrated that combining test biomarkers can improve the sensitivity and specificity of cholangiocarcinoma (CCA) diagnosis, thereby boosting their combined effective diagnostic capacity [97].

Annexin A1 (ANXA1)

ANXA1's role in cancer development and progression is complex and context-dependent. A simple classification of ANXA1 as solely a tumor suppressor or cancer-promoting factor is an oversimplification, as research has demonstrated its tumor-suppressive functions in certain neoplasms, yet its capacity to also facilitate oncogenic processes like proliferation, invasion, and metastasis in other carcinogenic contexts [98]. It's more likely a double-edged sword due to its function as tumor-suppressive or tumor-promoting effects, which makes it difficult to

classify it as simply one or the other [99]. As a result, the function of ANXA1 appears to differ in different types of cancer; for example, in melanoma and pancreatic cancer, ANXA1 is elevated and involved in the regulation of proliferation [100, 101]. It's known that the ANXA1 molecule was significantly and persistently upregulated during the long-term host-parasite interaction [102, 103].

In cholangiocarcinoma (CCA) patients, elevated expression levels of ANXA1 correlate with increased tumor stage, larger tumor size, and greater incidence of lymph node metastasis [104]. Both of which are traits of chronic opisthorchiasis. Hongsrichan, Rucksaken et al. (2013), have demonstrated that Annexin A1 (ANXA1) is the strongest immunohistochemical marker in distinguishing between cholangiocarcinoma (CCA) and hepatocellular carcinoma (HCC) in *O. viverrini*-infected hamsters. The study found that ANXA1 expression was significantly higher in hamsters with CCA induced by an *O. viverrini* infection and N-nitrosodimethylamine than in those with HCC and healthy liver tissues. The study also showed a high sensitivity (94%) and specificity (100%) of ANXA1 in distinguishing CCA from HCC, with a positive predictive value of 100% [105]. The findings of Kotepui et. al. (2022) strongly indicate that ANXA1 is a potential prognostic marker and may be used to screen tissues of CCA patients at risk of metastasis [106]. The examples above show that ANXA1 expression is involved in the carcinogenesis of chronic inflammation-related CCA, implying that it could be used to diagnose CCA. Hence, manipulation of ANXA1 action may be an alternative strategy to prevent metastasis of CCA. Nevertheless, the ability of ANXA1 to differentiate between Ov-CCA and non-Ov-CCA remains a critical question that requires further investigation.

Immunoglobulin G (IgG) and Immunoglobulin G4 (Ig4) antibodies

Infection with *O. viverrini*, a parasitic worm that affects the bile ducts, triggers the immune system to create antibodies to combat the parasite. Serological assays like ELISA can identify these antibodies even in blood or urine samples [107]. In *O. viverrini*, IgG antibodies have been demonstrated to have a 99.2% sensitivity and a 93% specificity for diagnosing *O. viverrini* infection [107], and it can persist in infected hosts even after being cured [108]. The close relationship between parasite-specific IgG and severe fibrosis suggests a specific immune response to the parasite [109]. A high prevalence of serum IgG for Ov-positive CCA patients and a correlation with overexpression of HER2 indicated poor survival of CCA [110]. Therefore, future clinical investigations of anti-HER2 therapies should also target Ov-CCA.

Specifically, Immunoglobulin G4 (IgG4) antibodies exhibit high specificity for *O. viverrini* infection and are absent in sera from healthy controls or individuals with other parasitic infections. Therefore, the presence of IgG4 antibodies in the serum or urine of an individual is a strong indicator of *O. viverrini* infection [110]. Elevated urine IgG to *O. viverrini* antigen significantly distinguishes individuals with APF and CCA, signifying its potential as a syndromic biomarker for estimating risk of renal and hepatobiliary pathologies in *O. viverrini* endemic areas [111]. However, it's important to note that the detection of IgG4 antibodies alone may not be sufficient to diagnose CCA or predict the risk of developing Ov-CCA. Additional diagnostic tests and risk assessments are needed to confirm the presence of CCA and assess the risk of developing CCA in patients with *O. viverrini* infection.

Oxidized alpha-1 antitrypsin (ox-A1AT)

Alpha-1 antitrypsin (AAT) is a significant protease inhibitor present in human blood (1-2 g/L), with oxidized alpha-1 antitrypsin (ox-A1AT) serving as a marker for oxidative stress [112, 113]. During an acute-phase reaction triggered by pro-inflammatory cytokines, such as IL-6, IL-8, IL-17, and TGF-β, alpha-1 antitrypsin (AAT) plasma levels can increase up to 2-4 fold above baseline, indicating its potential role in inflammation and oxidative stress in *O. viverrini* infection [114, 115]. Most of these pro-inflammatory cytokines are known to increase in opisthorchiasis and parasitic liver fluke that can increase oxidative stress in the liver by inducing chronic inflammation and producing reactive oxygen species (ROS) [115, 116].

The study by Jamnongkan and colleagues (2013) used indirect ELISA to evaluate the potential of serum ox-A1AT levels as a biomarker for detecting advanced periductal fibrosis (APF) and cholangiocarcinoma (CCA) in individuals infected with *O. viverrini*. The findings suggest that serum ox-A1AT levels have high sensitivity and specificity for identifying APF and CCA. At a cut-off value of 0.209, the sensitivity and specificity for identifying APF were 96.3% and 90%, respectively. At a cut-off value of 0.12, the sensitivity and specificity for identifying CCA were 81% and 80%, respectively. These results suggest that serum ox-A1AT levels have the potential as a biomarker for the early detection of APF and CCA in individuals infected with *O. viverrini* [117]. This study suggests that serum ox-A1AT level could be a potential predictive biomarker for detecting CCA and APF associated with *O. viverrini* infection. Furthermore, the study found that indirect ELISA was more sensitive (81%) than sandwich ELISA (35.7%) for detecting ox-A1AT in serum, which could be a useful screening method for routine serological testing/evaluation of ox-A1AT levels.

Plasma Checkpoint Kinase 1 (CHK1)

Checkpoint kinase 1 (CHK1) is a protein kinase crucial in the DNA damage response [118]. To preserve genomic fidelity, cells employ highly effective DNA damage repair systems that induce cell cycle arrest at key junctures such as the G1/S, intra-S, G2/M, and mitotic spindle checkpoints. These cycle control mechanisms, collectively termed cell cycle checkpoints, are critical for cell viability [119].

The *O. viverrini* infection promotes inflammation and results in DNA damage when combined with nitrosamine chemicals [120-122]. Analysis of the *O. viverrini*-infected plasma proteome revealed the presence of proteins with functions related to cell cycle regulation, cell proliferation, and cell signaling, such as Chk1, VCAM1, PIK3C2B, MAPK1, and PIM1, within the protein network [123].

Additionally, Phanaksri et.al.(2022), a study showed that the plasma checkpoint protein 1 (Chk1) is significantly increased in Ov-infected patients, with a sensitivity and specificity of 59.38% and 85.71%, respectively, using an OD450 cut-off of 0.6668, suggesting a possibility that DNA damage started during the infection period and may have continued during the development of CCA [123]. Higher plasma Chk1 levels may serve as a potential diagnostic biomarker for Ov-CCA. Some studies suggest that targeting Chk1 in DNA damage response (DDR) signaling pathways could offer therapeutic benefits, such as enhancing the activity of DNA-damaging agents, regulating cell cycle checkpoints, and modulating DNA repair and apoptotic events [124, 125]. Hence, there is potential for Chk1-targeting agents to also serve as therapeutic targets. However, current research exploring Chk1 in this capacity remains preliminary, particularly in Ov-CCA. Additional studies are necessary to confirm the suitability of Chk1 as a predictive or prognostic marker.

Opisthorchis viverrini granulin 1 (Ov-GRN-1)

Granulin, which has been implicated in the development of biliary tract cancer induced by liver fluke infection, exhibits potential utility as both a prognostic indicator and a therapeutic agent for wound healing [126]. The *O. viverrini* genome contains two genes encoding single granulin domains (Ov-GRN-1 and Ov-GRN-2) identified in in-silico generated ES products of *O. viverrini* [127]. Both *O. viverrini* and *C. sinensis* are recognized for their release of mitogenic and anti-apoptotic agents, including factors akin to granulin and thioredoxins. These substances promote cellular proliferation, resulting in the progression of cholangiocarcinoma [128-130].

Moreover, granulin functions as an extracellular growth factor, promoting cell proliferation and tumorigenesis. In a hamster model of *O. viverrini* infection and carcinogenesis, granulin expression was specifically identified in malignant cholangiocarcinoma (CCA) lesions, as demonstrated by immunohistochemistry, distinguishing it from normal and pre-cancerous biliary tissue [131]. Further analyses via Western blot and RT-PCR confirmed a notable increase in granulin levels within CCA specimens compared to non-cancerous liver tissue. This cancer-specific overexpression of granulin underscores its potential value as a biomarker, both within tissue samples and potentially in circulating blood.

Knocking out the Ov-GRN1-1 using CRISPR/Cas9 in a hamster model of liver fluke infection led to reduced fibrosis, biliary epithelial proliferation, and malignancy, resulting in less periductal fibrosis, fewer proliferating cholangiocytes, and lower expression of mutant p53 compared to control infections, underscoring the crucial role of Ov-GRN-1 in driving pathogenesis and cholangiocarcinogenesis during chronic *O. viverrini* infection [132]. Ov-GRN-1, secreted by *O. viverrini*, is detectable in host tissues and biofluids, with its levels correlating with infection intensity and severity of biliary abnormalities, indicating its potential as a diagnostic biomarker and prognostic indicator for Ov-CCA.

Recently, progranulin (PGRN) also has emerged as a noteworthy candidate for biomarker exploration in Ov-CCA [133, 134]. Ov-PGRN is secreted by the liver fluke and stimulates proliferation of host cholangiocytes and expression of inflammatory cytokines like IL-6 and IL-8, underscoring its role in pathogenesis [135]. Detection of Ov-PGRN levels and anti-Ov-PGRN antibodies may serve as biomarkers of infection intensity, hepatobiliary morbidity, and risk associated with *O. viverrini* infection.

Other markers with potential value

Early detection of CCA induced by *O. viverrini* is vital in resource-constrained regions with high prevalence, making any potential available biomarkers immensely valuable for public health. A study conducted by Aksorn and colleagues (2018) has shown that liver fluke infection-associated CCA can be differentiated from other forms of CCA using specific biomarkers, including immunoglobulin heavy chain, ALX1 (aristaless-like homeobox1 isoform X1), MICA (major histocompatibility complex polypeptide-related), translocated in liposarcoma (TLS), visual System Homeobox 2 (VSX2). These proteins have been identified in cholangiocarcinoma (CCA) cases associated with *O. viverrini* infection. However, these biomarkers' diagnostic capabilities must still be thoroughly characterized [25].

Other potential markers include the liver fluke protein 14-3-3 *O. viverrini* demonstrated that these proteins are numerous and constitutively expressed throughout the adult worm's life cycle and in most tissues [136]. The protein isoform 14-3-3 eta represents a potential screening and early diagnostic biomarker for Ov-CCA [137]. Additionally, the protein isoform 14-3-3 eta may be used as a screening and early diagnostic marker for Ov-CCA [138].

Cholangiocarcinoma associated with *O. viverrini* infection (Ov-CCA) displays distinct genetic, epigenetic, and transcriptional profiles compared to CCA not linked to this parasite [139]. While this study focused principally on potential molecular markers in Ov-CCA, the findings may also inform non-Ov-CCA cases, given the established role of *O. viverrini* infection as a risk factor for CCA pathogenesis. There are markers like (KLK11) expression, CD44v9, Ov IgG level, and CSC markers that can be used as the prognostic markers for CCA patients' survival [140, 141]. Although the paper itself does not directly analyze *O. viverrini* infection status or antibody levels (like Ov IgG) as a prognostic marker for CCA patient survival, the study provides evidence for CD44v9 and CSC markers as predictors of CCA patient survival, which can be applied in Ov-CCA as well.

There are several studies in the domain of non-Ov CCA where authors found that high expression was associated with tumor samples from CCA patients, lymph node metastasis, and poor overall survival in CCA patients. However, these studies do not address the potential influence of *O. viverrini* as a risk factor on their findings, leaving an opportunity for comparative investigations of such proteins in Ov-CCA and non-Ov-CCA.

Biomarker potential of MicroRNAs in Ov-CCA

Non-coding RNAs, especially miRNAs, are vital regulators in CCA, influencing cancer development

and recurrence through various cellular processes and epigenetic mechanisms [142, 143]. In CCA, an increasing number of miRNAs have been linked to the disease, and many of them have been characterized for their functional roles [144-146]. One noteworthy aspect of miRNAs in the context of Ov-CCA or even in non-Ov-CCA is their remarkable stability, even after formalin fixation and this stability has sparked substantial interest in utilizing miRNAs as potential biomarkers that can be explored using tumor biopsy samples preserved in formalin-fixed paraffin-embedded (FFPE) tumor blocks [147].

In *O. viverrini* infection leading to Ov-CCA, the expression of has-miR-192 was found to be elevated in both human and hamster liver tissues [148]. Examining distinct extracellular miRNA levels in plasma of *O. viverrini*-infected subjects compared to uninfected controls underscores their potential as non-invasive biomarkers. Circular RNAs (circRNAs) hold significant promise as markers for the initiation and advancement of CCA, presenting valuable prospects for diagnosis, therapeutic interventions, and prognostic surveillance of the disease [149, 150]. In 2015, a finding emerged from plasma miRNA profiling, revealing the induction of eight miRNAs, namely hsa-miR-885-5p, hsa-miR-505-3p, hsa-miR-483-5p, hsa-miR-92b-3p, hsa-miR-874, has-miR-1307-3, hsa-miR-1275, and hsamiR-320b, associated with *O. viverrini* induced intrahepatic cholangiocarcinoma (Ov-iCCA) from tissue and plasma, thus laying the foundation for a circulating miRNA-based biomarker panel for Ov-CCA [151].

miRNAs like Mir21 , miR-210, and 200 family (miR-200c, miR-200b, miR-200a, miR-429, and miR-141) [152] was observed in *O. viverrini*-Ov-iCCA and are dysregulated both in tumor tissue across histological subtypes and in plasma seem to have the most promising potential as diagnostic or prognostic biomarkers for Ov-induced ICC [153-155]. The miR-200 family serves as tumor suppressors, often reduced in cancer, with clinical significance for diagnostics, prognostics, and anti-cancer drug resistance [156] and miR-210 inhibition suppresses pro-inflammatory responses [157] and reduces the responsiveness of CCA cells to gemcitabine by suppressing HIF-3 (Hypoxia inducible factor 3), while concurrently sustaining HIF-1 activity [158, 159].

Elevated miR-21 serum levels were strongly associated with advanced clinical stage, increased invasion depth, lymph vessel infiltration, metastasis, poor differentiation, non-resectable status, and poorer survival in CCA patients [160]. A secreted miR-21 can be detected in blood and body fluids and may serve as a non-invasive biomarker and may distinguish metastatic versus non-metastatic tumors [161]. It would be interesting to investigate if similar correlations exist in ICC induced by O. viverrini.

Certain miRNAs and lncRNAs show potential in discerning *O. viverrini* from other parasites. Conserved miRNAs with crucial functions and their extracellular release warrant deeper exploration as biomarkers for diagnosis, prognosis, or therapeutic response [162]. Further validation of miRNA biomarkers and functional characterization of their mechanisms in Ov-CCA may aid prognosis, treatment monitoring, and the development of RNA-targeted therapeutics for this cancer etiology.

Limitations in the Discovery and Clinical Implementation of Biomarkers for Ov-CCA

The discovery and implementation of biomarkers for Ov-CCA in clinical trials and field studies face several challenges. Despite many publications on biomarker discovery, only a few get validated for clinical use due to poor reproducibility and lack of standardization in specimen collection [163, 164]. In our findings, we found few studies that have evaluated the same biomarker candidates, making it difficult to compare results across studies and validate utility. There was a lack of consistent biomarker panels being assessed. The majority of experiments designed to confirm preliminary biomarkers rely predominantly on the hamster model of Ov-CCA. Further efforts utilizing well-defined biomarker candidates probed across multiple human sample cohorts will be imperative to substantiate the veracity and reproducibility of proposed markers. Nevertheless, the top biomarker candidates emerging from our review were selected based on proper evaluation of sensitivity, specificity, and other test accuracy measurements from a single robust study. For example:

• CCA-associated carbohydrate antigen (CCA-CA) demonstrated high sensitivity (87.6%) and specificity (89.6%) in multiple studies for diagnosis and prognosis.

• IL-6 showed good sensitivity (80%) and specificity (90%) for predicting prognosis in several human studies.

• IgG4 antibodies revealed excellent specificity (99.2%) and sensitivity (93%) for Ov-CCA.

• Anti-Hsp90α antibodies exhibited sensitivity of 76.2% and specificity of 71.4% in a well-designed study.

In contrast to many existing discovery-based investigations, our approach was centered on candidates demonstrating both accuracy and reliability. However, additional validation could strengthen its inclusion as a component of a biomarker panel.

In addition, more than the limited availability of clinical specimens and small sample sizes, especially in resource-limited settings, limits statistical power in demonstrating associations between biomarkers and conditions [165]. Besides regulatory hurdles (absence of a specific FDA-approved biomarker), financial constraints, technical limitations, and the disease's complexity are the major reasons behind these challenges. Another major challenge in biomarker discovery for Ov-CCA infection is the host's immune response variability, which can lead to difficulty generalizing the biomarker results. Furthermore, co-infections with other parasites can complicate the interpretation of biomarker results [166, 167]. Therefore, identifying a single biomarker alone may lack the requisite diagnostic or prognostic sensitivity and specificity for Ov-CCA. Rather, combining multiple biomarkers related to inflammation, cell cycle control, and DNA damage - known to be upregulated in Ov-CCA versus healthy tissue - should be investigated to enhance early CCA detection and prognosis. When developing a biomarker for Neglected Tropical Diseases (NTDs) such as *O. viverrini*, it is crucial to consider the cost-effectiveness of the test, as this can significantly

impact its utilization in resource-limited settings [168]. To address these challenges, collaborative efforts are required from researchers, funding agencies, and policy-makers to prioritize research on CCA biomarkers caused either by parasite infection or other etiologies.

Immunoproteomics to Discover and Validate Biomarkers and Targets for O. viverrini

Among the numerous options for assessing biomarker validity, immunoproteomic profiling represents a promising conventional approach for efficacy testing due to its capacity for high-throughput and sensitive antigen characterization and cost-effectiveness to validate putative biomarkers. Given the prevalence of Ov-CCA in lower-income populations, the use of immunoproteomics ensures that biomarker validation can be conducted using standard equipment and methods readily available to scientists in developing regions, promoting equity in healthcare research. Additionally, the capacity for multiplexing and automation within immunoproteomic pipelines further enhances its suitability for robust biomarker verification in resource-limited settings. Immunoproteomic assay integrates antibody specificity and mass spectral analysis, enabling sensitive and efficient detection of specific antigens or antibodies [169]. Robust validation of antibody biomarkers can be achieved by employing antibody-based assays such as ELISA or protein arrays across substantial patient cohorts and matched controls. Meanwhile, analysis of protein biomarkers using techniques including 2D gels, western blots, and LC-MS/MS enables quantification of differentially expressed proteins to reveal novel candidate biomarkers warranting further scrutiny. Furthermore, incorporating animal profiling techniques and human tissue samples may provide valuable insights into identified biomarkers' translational potential and biological relevance [170, 171].

Additionally, optimization and clinical validation of this biomarker panel enable the development of a multiplex assay for rapid and efficient detection in clinical practice. This confirmatory approach strengthens biomarker development pipelines by analytically validating identified leads, enhancing biomarker discoveries for *O. viverrini* infection and complementing existing biomarkers (Figure 2). Nevertheless, it also uncovers stage-specific diagnostic biomarkers, identifies novel antigens associated with parasite maturation and virulence, and enables precise tracking of post-treatment outcomes. This technique accurately maps antibody responses against parasite antigens expressed throughout different developmental stages.

Immunoproteomic profiling of *O. viverrini* infections allows for identifying antigens specifically upregulated in juvenile stages, aiding in understanding parasite development and identifying potential early markers. Comparative analysis across different host species provides insights into proteins critical for parasite maturation and survival [172, 173]. This approach aids in discovering crucial parasite proteins, thus facilitating the development of targeted interventions for controlling and managing opisthorchiasis.

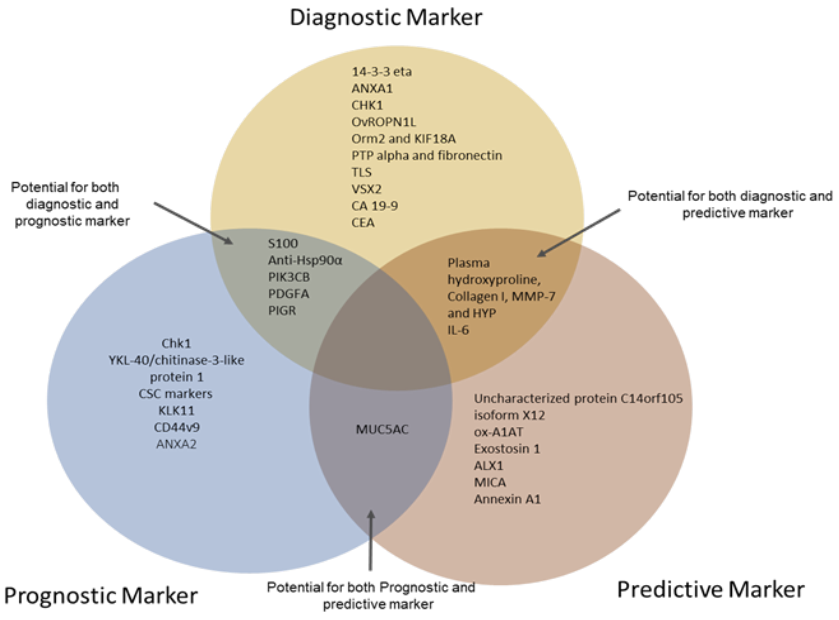

Figure 2. Venn Diagram Representation of Notable Biomarker Proteins. PIK3CB, Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta; Chk1, Checkpoint kinase 1; YKL-40, Chitinase-3-like protein 1; CSC markers, Cancer stem cell markers; PDGFA, Platelet-derived growth factor subunit A; KLK11, Kallikrein-11; CD44v9, CD44 variant 9; IL-6, Interleukin 6; Anti-Hsp90α, Antibodies against Heat shock protein 90 kDa alpha; ANXA1 and ANXA2, Annexin A1 and Annexin A2; ox-A1AT, Oxidized alpha-1-antitrypsin; Exostosin 1: Exostosin glycosyltransferase 1; OvROPN1L, Rhophilin associated tail protein 1; Ov-TSP1, *Opisthorchis viverrini* tetraspanin-1; Plasma hydroxyproline, Collagen I, MMP-7, and HYP, Plasma hydroxyproline, Collagen type I, Matrix metallopeptidase 7, Hydroxyproline, 14-3-3 eta: 14-3-3 protein eta, Orm2 and KIF18A, Orosomucoid 2 and Kinesin family member 18A PTP alpha and fibronectin; PIGR, Polymeric immunoglobulin receptor; TLS, Translocated in liposarcoma; VSX2, Visual system homeobox 2; S100: S100 calcium-binding protein; MUC5AC, Mucin-5AC.

Conclusion: Future Directions in CCA Biomarker Research

A comprehensive understanding of the disease pathogenesis and underlying mechanisms is essential for identifying biomarkers that can effectively reflect the disease status or predict disease progression. Given the lack of widely accepted or clinically validated prognostic and predictive biomarkers for CCA and Ov-CCA, discovering minimally invasive indicators is imperative.

By compiling the current evidence and identifying the lead biomarkers supported by the strongest validation data, this review provides clarity on the current state of usable biomarkers for Ov-CCA. However, rigorous validation and assessment of these preliminary biomarkers in large prospective clinical studies are essential before considering practical clinical implementation. Furthermore, since carcinogenesis is a multi-path, complex process, it may take more than one biomarker to correlate with a tumor appropriately. Hence, high-throughput technologies for omics data allow for assessing hundreds or more potential biomarker candidates for specific diseases or disease states.

Immunoproteomic profiling demonstrates biomarker potential, yet to comprehensively understand the complexity of biological systems, integrating additional omics approaches such as genomics, proteomics, metabolomics, and transcriptomics is necessary for a comprehensive view [174, 175]. Single-cell multiomics, mass spectrometry-based proteomics, Next-generation sequencing (NGS), metabolomics, and integration of multiomics data with advanced computational methods provide comprehensive insights into cellular heterogeneity, disease mechanisms, protein biomarkers, genetic variants, gene expression patterns, metabolic pathways, and potential therapeutic targets [175, 176].

Studies analyzing large amounts of biological data to identify patterns and changes in gene expression, protein production, metabolic pathways, and other molecular processes associated with a particular disease or condition can enhance the quality of immunoproteomic profiling [177]. For massive data like such, pilot studies can be conducted to assess the potential of discovering biomarkers, given the limited availability of participants and high costs. For instance, HiPerMAb, a computational tool, can be employed to evaluate pilot studies by calculating performance measures and comparing the number of "good" biomarker candidates with expected values, even when statistical tests fail to provide significance [178]. This approach can help determine the feasibility of conducting large-scale biomarker discovery studies and inform the design of subsequent trials. HiPerMAb can help evaluate numerous CCA biomarkers that require careful evaluation.

Additionally, machine learning (ML) techniques can help identify potential biomarkers for *O. viverrini* by analyzing various data types, such as gene expression patterns and serum samples. ML algorithms can help reduce the dimensionality of large datasets, select relevant features, and integrate different datasets to enhance the predictive model's performance [179]. Furthermore, various machine learning algorithms, such as random forests, support vector machines, and neural networks, can be used for biomarker discovery and validation [180]. By applying ML algorithms to *O. viverrini* biomarkers, researchers can potentially identify specific genetic variations that distinguish individuals with Ov-CCA from those without it, leading to the development of new diagnostic tools and therapies for the disease.

Author Contribution Statement

Alok Kafle conducted the literature search, data extraction, and data analysis and Sutas Suttiprapa provided critical feedback and guidance throughout the review process.

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Data Availability

As this is a review paper summarizing and analyzing existing literature, there is no original data associated with this manuscript.

Study Registration

This review was conducted following the PRISMA guidelines for systematic reviews. Although the study was not formally registered, we systematically collected all relevant papers pertaining to biomarker-based studies related to Ov-CCA using the Covidence platform, ensuring a comprehensive and rigorous review process.

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