HHS Public Access

Author manuscript

Int J Infect Dis. Author manuscript; available in PMC 2022 October 15.

Published in final edited form as:

Int J Infect Dis. 2022 March ; 116: 80–84. doi:10.1016/j.ijid.2021.12.347.

Rapid assessment of *Opisthorchis viverrini* IgG antibody in serum: A potential diagnostic biomarker to predict risk of cholangiocarcinoma in regions endemic for opisthorchiasis

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Abstract

Background: Opisthorchiasis is caused by an infection with fish-borne liver flukes of the genus *Opisthorchis*. Opisthorchiasis frequently leads to chronic inflammation in the biliary tract and is classified as a group 1 biological carcinogen by the International Agency for Research on Cancer: a definitive risk for cholangiocarcinoma (CCA).

Methods: We used the rapid immunochromatographic test (ICT) to detect anti-*Opisthorchis viverrini* IgG and IgG4 subclass antibodies in sera of patients with CCA. The ICT kits were developed based on soluble antigens excreted and secreted by *O. viverrini* adult worms.

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Conceptualization and design: RR, VL, WS, AK, KP, WI, VHM, PMI, PJB, and WM. Methodology: RR, VL, LS, WS, AK, KP, OS, PMI, and PJB. Formal analysis and investigation: RR, VL, LS, WS, AK, KP, OS, PMI, and WM. Writing-original draft preparation: RR, VL, WI, and VHM. Writing-review and editing: RR, WI, PJB, PMI, and WM. Supervision: WM, VHM, PJB, and PMI. All authors read and approved the final manuscript.

Ethics

This study was approved by the research ethics committee of the Khon Kaen University Ethics Committee for Human Research (Ethics number: HE641114, approved June 7, 2021).

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2021.12.347.

Results: ICT indicated sera was positive for IgG and IgG4 antibodies, respectively, in 22 (61.1%) and 15 (41.6%) participants of the 36 study participants diagnosed with CCA ($P > 0.05$). Our study also included groups with other cancers and with liver cirrhosis, where the IgG ICT and IgG4 ICT kits were 27.7% (13/47) and 25.5% (12/47) positive, respectively ($P > 0.05$). Neither total the IgG ICT nor the IgG4 ICT yielded positive results in a control group of 20 healthy participants. Moreover, the percentage positivity rate using the ICT for total IgG between the CCA group and the other cancers and liver cirrhosis group was significantly different ($P < 0.05$). By contrast, no significant difference between these groups was apparent in the ICT for IgG4 antibody. The CCA group was 6.53 times more likely to have positive anti–*O. viverrini* IgG antibody (odds ratio 6.53, $P < 0.001$) and 3.27 times more likely to have positive anti–*O. viverrini* IgG4 antibody (odds ratio 3.27, $P = 0.010$) than the non-CCA group.

Conclusion: This information is of potential value for the development of a diagnostic biomarker to predict risk for *O. viverrini* infection-associated CCA.

Keywords

Cholangiocarcinoma; Rapid serodiagnosis; Opisthorchis viverrini; Opisthorchiasis; Helminthiases-associated cancer; Diagnostic biomarker

Introduction

Cholangiocarcinoma (CCA) is a malignant neoplasm of the bile duct. This cancer has been classified as either intrahepatic or extrahepatic, with the location in the second-order bile ducts as the separating point. CCAs can be distinguished on the basis of their anatomic location along the biliary tree and categorized into the following three types depending on clinical presentation: intrahepatic CCA (iCCA), perihilar CCA (pCCA), and distal CCA (dCCA) (Blechacz et al., 2011; Deoliveira et al., 2011; Waseem and Tushar, 2017). This malignancy is usually difficult to diagnose until it becomes advanced or disseminated and has a poor prognosis with a 5-year survival rate of approximately 5% for iCCA and 17% for pCCA and dCCA (Deng et al., 2021; Squadroni et al., 2017). Prevalence varies greatly worldwide. The northeastern provinces of Thailand have the highest prevalence of CCA, with more than 80 cases per 100,000 people affected (Banales et al., 2016; Kamsa-ard et al., 2018; Squadroni et al., 2017). In regions where opisthorchiasis is endemic, including the lower Mekong River subregion (i.e., Cambodia, Lao People's Democratic Republic, southern Vietnam, Myanmar, and Thailand) (Sripa et al. 2010; Aung et al. 2017), more than 10 million people are estimated to be infected with *O. viverrini*. Infection results from the ingestion of raw or semicooked freshwater cyprinid fishes carrying the larval stage of the parasite called metacercaria. After ingestion, the motile juvenile fluke excysts and enters the common bile through the ampulla of Vater and matures to the adult form of liver fluke in the intrahepatic bile ducts. These parasites live in the biliary tract for several years, laying eggs, which in turn pass into the bile and eventually exit the host through feces (Sripa et al. 2010). The risk factors for opisthorchiasis-related CCA include chronic inflammation and attendant injury of the biliary epithelium due to persistent parasitism by the flukes (Brindley et al., 2015, 2021). The high incidence of CCA in regions where liver fluke occurs likely involves risk factors unique to this tropical disease, primarily infection with *Opisthorchis*

viverrini, which is classified as a group 1 biological carcinogen by the International Agency for Research on Cancer (IARC, 2012; Khuntikeo et al., 2015; Sripa et al., 2011).

In a 2015 CCA screening project of 47,258 people residing in the northeastern region of Thailand, 42.2% were infected with *O. viverrini* due to the consumption of uncooked fish, and 29.9% were diagnosed with CCA (Khuntikeo et al., 2015). Infection with this helminth induces biological and chemical effects on host tissues–promoters of DNA lesions, which result in chronic inflammation, fibrosis, and other changes in microenvironment of the hepatobiliary tract (Brindley and Loukas, 2017; Gouveia et al., 2017). One hypothesis suggests that reactive metabolites of oxysterol-like precursors of *O. viverrini* and *O. felineus* are initiators of carcinogenesis, representing genotoxins that mutate genes of the epithelial cells that line the bile ducts (Brindley et al., 2015; Gouveia et al., 2017).

Stool examination to detect eggs in feces is the gold standard for the diagnosis of *O. viverrini* infection. This technique can diagnose patients with heavy active infection, but false negatives occur in latent infections with low worm burdens or biliary obstruction (Phupiewkham et al., 2021; Sawangsoda et al., 2012). Serodiagnosis for opisthorchiasis also can provide sensitive and specific diagnosis (Phupiewkham et al., 2021; Sadaow et al., 2019; Tesana et al., 2007). Serum immunoglobulin G (IgG) antibody against antigens in lysates of adult worms and of metacercariae can persist in infected hosts after parasitologic cure (Akai et al., 1995). Moreover, the immunoglobulin G4 subclass (IgG4) antibody is useful and specific for diagnosis of opisthorchiasis (Phupiewkham et al., 2021; Tesana et al., 2007). It is noteworthy that serum titers of anti–*O. viverrini* antibodies have been found to be higher in cases of CCA than in cases of cholangitis caused by *O. viverrini* or in the absence of infection (Akai et al., 1994; Itoh et al., 1994). Accordingly, serum IgG and IgG4 antibodies can be of value not only for the detection of *O. viverrini* infection but also in screening for *O. viverrini*–associated CCA.

Recently, Sadaow et al. (2019) developed the rapid diagnostic immunochromatographic test (ICT) as a point-of-care (POC) testing tool for IgG antibody detection, which can be used at the bedside without the need for sophisticated equipment. Here, we tested this ICT for the detection of anti–*O. viverrini* IgG and IgG4 subclass antibodies in the serum of study participants with CCA. Our present findings confirm that this ICT may be able to augment the diagnosis of liver fluke infection by stool examination. It also may be useful to increase prediction and awareness of the new *O. viverrini*–associated CCA cases in at-risk populations and to reduce the costs of screening, thereby enabling better planning both to reduce risk and also increase survival outcomes for opisthorchiasis-associated CCA.

Materials and methods

Sera

A total of 103 serum samples from the Frozen Serum Bank at the Faculty of Medicine, Khon Kaen University were included in this study. These sera were assigned to three groups as follows: group I, CCA group (n = 36); group II, other cancers and liver cirrhosis group (n = 47) including hepatocellular carcinoma (n = 28), liver cirrhosis (n = 4), colorectal cancer (n = 8), and brain cancer (n = 7); and group III, other healthy individuals (n =

20). Persons who donated the sera in this latter control group were free of malignancies and also free of intestinal parasites as ascertained by parasitologic methods (Elkins et al., 1986) at the time of phlebotomy. Demographic data are provided in Table 1. The residual sera, investigated here, were obtained from each participant in this study before surgery at Srinagarind Hospital, Khon Kaen University.

Detection of immunoglobulin G by rapid diagnostic ICT

The opisthorchiasis and clonorchiasis diagnostic ICT kit using *O. viverrini* excretory-secretory (ES) antigen (Sadaow et al., 2019) was used for screening of IgG antibody against *O. viverrini* in human serum samples. The ICT kit was used as described (Sadaow et al., 2019). Briefly, 5 μL of diluted serum (1:30) was dispensed into the sample well, followed by 90 μL of running buffer. The assay result was read at 15 minutes after the addition of the running buffer; if red bands appeared at both test and control lines, serum was considered to be positive, whereas if a red band appeared only at the control line, serum was considered to be negative. The intensity of the bands was scored visually by comparison with the reference card. A band intensity ≥ 0.5 was confirmed to be positive (Sadaow et al., 2019).

Detection of IgG4 subclass antibody by rapid diagnostic ICT

For the detection of serum IgG4 antibody against *O. viverrini*, the newly developed ICT strip under optimal condition was used as described (Sadaow et al., 2019) with some modification. Briefly, 2 mg/mL of *O. viverrini* ES antigen (Intapan and Maleewong, 2006; Sadaow et al., 2019) and 2 mg/mL of anti-mouse IgG (Lampire Biological Laboratories, Pipersville, Pennsylvania) were dispensed onto nitrocellulose membrane (Sartorius Stedim Biotech SA, Goettingen, Germany), at a flow rate of 0.1 μL/mm, to serve as the test and control lines, respectively. Six micrograms per milliliter of mouse anti-human IgG4 (Invitrogen, Eugene, Oregon) conjugated with colloidal gold (Kestrel BioSciences Co., Pathumthani, Thailand) was sprayed onto a glass microfiber filter GF33 (conjugate pad) (Whatman Schleicher & Schuell, Dassel, Germany). For testing, 5 μL of undiluted serum samples was added to a sample well, followed by 100-μL running buffer. The assay result was read at 15 minutes after the addition of the running buffer by visual examination: the intensity of the band was scored visually by comparison with the reference card. A band intensity ≥ 0.5 was considered to be positive.

Statistical analysis

The Shapiro-Wilk test was used to assess data normality. Pearson chi-square test was used to investigate the associations between categorical variables between groups. The correlations between results of IgG and IgG4 ICT kits were tested using the McNemar test statistic. The odds ratios with their 95% confidence interval (CI) and *P* value were obtained using logistic regression. A *P* value ≤ 0.05 was considered statistically significant. Statistical analyses were performed by using STATA version 10.1 (Stata Corp, College Station, Texas).

Results

We used the ICT kits for detection of anti–*O. viverrini* IgG and anti–*O. viverrini* IgG4 antibodies. For 36 CCA samples, the IgG ICT kit showed a positivity of 61.6% in 22 cases

and positivity by IgG4 ICT kit in 15 cases (41.6%) (P value > 0.05) (Table 2). For 47 other cancers and liver cirrhosis samples, the IgG ICT showed a positivity in 13 cases (27.7%) and the IgG4 ICT showed 25.5% (12 participants) positivity (P value > 0.05) (Table 2). Positivity of CCA samples in both IgG and IgG4 ICT kits was 12 of 36 cases (33.3%), whereas only 10 (27.8%) and 3 (8.3%) cases were positive with IgG ICT and IgG4 ICT kits, respectively. When the positive results of both methods were combined, the sensitivity increased to 69.4% (25/36). Of 47 cases, 8 samples (17%) from the other cancers and liver cirrhosis group were positive in both IgG and IgG4 ICT kits (Supplementary Table 1). Neither IgG ICT nor IgG4 ICT yielded positive results in the 20 healthy controls or in 4 liver cirrhosis cases. The number of IgG antibody–positive sera was significantly different between the CCA group and the other cancers and liver cirrhosis group (P value < 0.05). However, there was no significant difference (P value > 0.05) in the IgG4 antibody detection (Figure 1). In regression analyses, the CCA group was significantly more likely to include total IgG antibody (odds ratio 6.53, $P \leq 0.001$) and more likely to include IgG4 antibody (odds ratio 3.27, $P = 0.010$) than the non-CCA groups (i.e., the other cancers and liver cirrhosis group and the healthy control group) (Table 3).

Discussion

Whereas the detection of the eggs of *O. viverrini* in the stool is the gold standard for the diagnosis of opisthorchiasis, detection of *O. viverrini* eggs in stool samples can be hampered by obstructive jaundice during CCA. Thus, several reports have established that in the setting of active *O. viverrini* infection during CCA, the fecal *O. viverrini* egg count and estimated incidence of CCA was only poorly correlated (Sriamporn et al., 2004; Srivatanakul et al., 1991). Likewise, fecal egg numbers may only be weakly correlated with antibody (Phupiewkham et al., 2021). Nonetheless, other studies in Thailand have revealed that the levels of specific anti–*O. viverrini* antibodies in human sera as examined by ELISA and the incidence of CCA were positively correlated (Akai et al., 1994; Itoh et al., 1994). Within this context, our present study focused on the examination of IgG and IgG4 antibodies against *O. viverrini* in serum during CCA by using rapid POC diagnostic ICT kits.

For the CCA group, anti-IgG *O. viverrini* antibody revealed a positivity rate of 61.1%, which was in line with previous findings for CCA sera that used anti-IgG antibody against somatic antigens of adult *O. viverrini* flukes (Itoh et al., 1994; Titapun et al., 2020). The detection rate for parasite-specific anti-IgG4 antibody was lower (41.6% positivity). This outcome conforms with our recent findings for residents in an opisthorchiasis-endemic region of rural Khammouane Province in central Lao PDR, which suggests that this anti-IgG–based test performs better than the anti–*O. viverrini* IgG4 detection for the diagnosis of fluke infection (Phupiewkham et al., 2021). Interestingly, when considering the combined findings of both methods, the sensitivity of antibody detection increased to 69.4% (25/36), and hence, both biomarkers can be used to expand capacity screening to predict risk of opisthorchiasis-related CCA.

It is notable that about 1 in 4 participants in the group that includes other cancers and liver cirrhosis was positive for anti–*O. viverrini* IgG (27.7%) and IgG4 (25.5%) antibodies,

whereas more than a third of the participants from the hepatocellular carcinoma group was seropositive for anti–*O. viverrini* ES IgG4 antibody (39.3%). The parsimonious interpretation of these findings was that these seropositive results reflected historical exposure to infection, given that these participants lived in the opisthorchiasis-endemic region of Thailand.

These findings also revealed a significant positive association between CCA and anti–*O. viverrini* IgG antibody. Thus, there was a greater likelihood of *O. viverrini* antibody using IgG ICT and IgG4 ICT kits in the participants with CCA than in participants without CCA, which conforms with the earlier reports (Itoh et al., 1994). Additional support was shown by the presence of anti–*O. viverrini* IgG antibody and the expression of growth factor receptor (HER2 protein) associated with poorer prognosis of CCA (Titapun et al., 2020). Opisthorchiasis is a definitive risk for CCA; in addition to the application of guidelines of the Thai National Health Control Program and the Cholangiocarcinoma Screening and Care Program (Khuntikeo et al., 2015, 2016 ,), serodiagnosis can also provide sensitive alternative techniques for the detection of this infection (Akai et al., 1994; Itoh et al., 1994; Sadaow et al., 2019; Tesana et al., 2007; Titapun et al., 2020).

In conclusion, these new findingshighlighted the diagnostic potential of a rapid ICT platform that uses ES products from *O. viverrini* flukes for serodiagnosis of opisthorchiasis-associated CCA. People living in areas endemic for opisthorchiasis can be rapidly screened for liver fluke infection using the rapid IgG ICT and IgG4 ICT kits, including where infections may be asymptomatic or mild, and where there are complications that can include hepatobiliary morbidity involving hepatomegaly, cholangitis, cholecystitis, periductal fibrosis, and/or gallstones along with a stool examination that is negative for *Opisthorchis* eggs (Phupiewkham et al., 2021; Sawangsoda et al., 2012). The information is of note with respect to prognostic and diagnostic biomarkers for screening for those at risk of developing CCA in regions endemic for liver fluke infection and for disease prevention. However, other associations and factors including dietary nitrosamines, interactions or changes in the gastrointestinal and biliary microbiota, primary sclerosing cholangitis, inflammatory bowel disease, metabolic syndromes, viral hepatitis, and carriage of species of *Helicobacter* also need to be considered (Brindley et al., 2015).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We express our gratitude to Jitaporn Harasan from the Mekong Health Science Research Institute for provision of expert immunochromatographic technique.

Funding

This study was supported by Distinguished Research Professor Grant, Thailand Research Fund (TRF) [PMI and WM, grant no. DPG 6280 0 02]; Khon Kaen University, Research and Graduate Studies Affairs [OS, PMI, and WM, Research Program]; Faculty of Medicine, Khon Kaen University [PMI and WM, grant no. DR63101 and RG63301]; a scholarship under the Post-Doctoral Training Program from Khon Kaen University (KKU) [RR, grant no. PD256409]; and the National Cancer Institute, National Institutes of Health (NIH), USA [PJB and PMI, award

no. RO1CA164719]. The contents of this report are solely the responsibility of the authors and do not necessarily represent the official views of the TRF, KKU, or the NIH.

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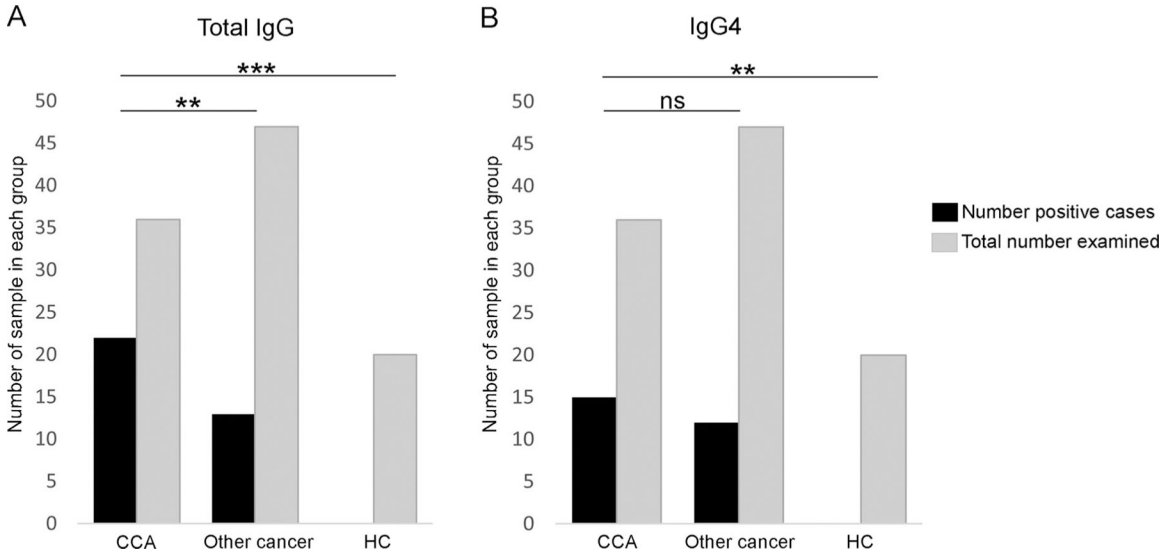

Figure 1.

Rates of positive detection of anti–*O. viverrini* IgG (A) and IgG4 antibodies (B) with the total IgG ICT and IgG4 ICT kits. Statistically significant differences between CCA, other cancer and liver cirrhosis, and healthy control groups are indicated by asterisks: ***$P \leq$ 0.001; **$P \leq$ 0.01; ns, not significant. CCA, cholangiocarcinoma sera (n = 36); Other cancers, other cancers and liver cirrhosis sera (n = 47); HC, healthy control sera (n = 20); Pearson chi-square test.

Table 1

Demographic and baseline characteristics of the study participants.

Characteristics	CCA (n = 36)	Other cancers and liver cirrhosis (n = 47)	Healthy control (n = 20)	*P* value[a]
Gender				0.385
Male, n (%)	24 (66.7)	34 (72.3)	11 (55.0)	
Female, n (%)	12 (33.3)	13 (27.7)	9 (45.0)	
Age (year)				0.122
≤ 40, n (%)	2 (5.6)	6 (12.8)	4 (20.0)	
41–50, n (%)	1 (2.8)	5 (10.6)	4 (20.0)	
51–60, n (%)	16 (44.4)	12 (25.5)	6 (30.0)	
>60, n (%)	17 (47.2)	24 (51.1)	6 (30.0)	
Mean ± SD (range)	59.7 ± 9.8 (32–77)	56.7 ± 15.0 (15–79)	50.1 ± 12.9 (27–65)	
Location of CCA tumor[b]				
Distal, n (%)	4 (11.1)			
Intrahepatic, n (%)	17 (47.2)			
Perihilar, n (%)	14 (38.9)			
Mixed location, n (%)	1 (2.8)			
Gross pathology of CCA				
Intraductal growth, n (%)	9 (25.0)			
Mass-forming type, n (%)	14 (38.9)			
Periductal infiltrating, n (%)	13 (36.1)			

[a] *P* value for test of difference between-group was compared using Pearson's chi-squared test.

[b] Anatomic locations of CCA were differentiated as described (Blechacz et al., 2011).

Table 2

Reactive frequencies of *Opisthorchis viverrini* antibody using total IgG ICT and IgG4 ICT.

	Number of positive cases (%) IgG ICT		*P* value[a] IgG4 ICT
Cholangiocarcinoma			
Overall (n = 36)	22 (61.1)	15 (41.6)	0.092
Distal (n = 4)	3 (75.0)	2 (50.0)	
Intrahepatic (n = 17)	10 (58.8)	5 (29.4)	
Perihilar (n = 14)	8 (57.1)	7 (50.0)	
Mix (n = 1)	1 (100.0)	1 (100.0)	
Other cancers and liver cirrhosis			
Overall (n = 47)	13 (27.7)	12 (25.5)	1.000
Hepatocellular carcinoma (n = 28)	9 (32.1)	11 (39.3)	
Liver cirrhosis (n = 4)	0 (0)	0 (0)	
Colorectal cancer (n = 8)	3 (37.5)	1 (12.5)	
Brain cancer (n = 7)	1 (14.3)	0 (0)	

[a]Exact McNemar significance probability

Table 3

The statistical analysis showing the odds ratios and 95% confidence interval among the CCA group, other cancers and liver cirrhosis group, and the healthy control group.

	Odds ratio			
	IgG (95% CI)	***P* value**	**IgG4 (95% CI)**	***P* value**
CCA vs other cancers and liver cirrhosis (n = 83)	4.11 (1.63–10.37)	0.002*	2.08 (0.82–5.29)	0.121
CCA vs non-CCA[a] (n = 103)	6.53 (2.66–16.10)	<0.001*	3.27 (1.32–8.14)	0.010*

[a]Value calculation when combining non-CCA (other cancers and liver cirrhosis and healthy control) groups.

*Statistically significant difference, $P \leq 0.05$