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นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

การตรวจวัดแอนติบอดีต่อแอนติเจนของพยาธิใบไม้ตับ *Opisthorchis viverrini* ในตัวอย่างซีรัมของผู้ป่วยมะเร็งท่อน้ำดี

พงศ์ศรัณย์ กิมาวหา[1,2,4], อภิญญา จุ๊สกุล[2,4], ไพบูลย์ สิทธิถาวร[3,4], อัญชลี เตชะเสน[2,4*]

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บทคัดย่อ

ประชากรมากกว่า 8 ล้านคนที่อาศัยอยู่ในพื้นที่ภาคตะวันออกเฉียงเหนือของประเทศไทยมีอัตราการติดเชื้อพยาธิใบไม้ตับ *Opisthorchis viverrini* (Ov) สูง ซึ่งเป็นสาเหตุสำคัญที่ก่อให้เกิดโรคมะเร็งท่อน้ำดี โดยพบว่าพยาธิใบไม้ตับสามารถกระตุ้นให้เกิดกระบวนการอักเสบแบบเรื้อรังและการตอบสนองทางภูมิคุ้มกันนำไปสู่การเป็นมะเร็งท่อน้ำดี การศึกษานี้มีวัตถุประสงค์เพื่อตรวจวัดระดับของแอนติบอดีต่อแอนติเจนของพยาธิใบไม้ตับ Ov ในซีรัมของผู้ป่วยมะเร็งท่อน้ำดีเทียบกับกลุ่มคนที่มีผลอัลตราซาวด์ช่องท้องปกติโดยใช้เทคนิคอีไลซา และศึกษาความสัมพันธ์ระหว่างผลการตรวจวัดแอนติบอดีกับผลการตรวจทางพยาธิวิทยาคลินิกของผู้ป่วย จากจำนวนตัวอย่างผู้ป่วยมะเร็งท่อน้ำดี 45 รายและกลุ่มคนปกติ 45 ราย พบว่าให้ผลบวกต่อการตรวจวัดแอนติบอดีต่อแอนติเจนของพยาธิใบไม้ตับ Ov คิดเป็น 35.6% และ 53.3% ตามลำดับ นอกจากนั้นในกลุ่มผู้ป่วยมะเร็งท่อน้ำดีที่ให้ผลบวกต่อการตรวจวัดแอนติบอดีมีความสัมพันธ์อย่างมีนัยสำคัญทางสถิติกับความรุนแรงของระยะมะเร็งท่อน้ำดี (p=0.036) และมีแนวโน้มสัมพันธ์กับอัตราการรอดชีวิตที่ต่ำของผู้ป่วย ทั้งนี้ไม่พบความแตกต่างของระดับของแอนติบอดีในกลุ่มผู้ป่วยมะเร็งท่อน้ำดีและกลุ่มคนปกติ โดยสรุป จากการศึกษานี้พบว่าแอนติบอดีต่อแอนติเจนของพยาธิใบไม้ตับ Ov ในซีรัมยังไม่สามารถใช้เป็นตัวบ่งชี้ทางชีวภาพในการวินิจฉัยกลุ่มผู้ป่วยมะเร็งท่อน้ำดีได้อย่างมีประสิทธิภาพ แต่อย่างไรก็ตามพบความสัมพันธ์ระหว่างระดับของแอนติบอดีกับความรุนแรงของระยะมะเร็งท่อน้ำดี ซึ่งต้องทำการศึกษาในเชิงลึกต่อไป

คำสำคัญ: *Opisthorchis viverrini*, โรคมะเร็งท่อน้ำดี, อีไลซา, Arbitrary unit

[1]ชีวเวชศาสตร์ บัณฑิตวิทยาลัย มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น
[2]คณะเทคนิคการแพทย์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น
[3]ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น
[4]สถาบันวิจัยมะเร็งท่อน้ำดี มหาวิทยาลัยขอนแก่น จังหวัดขอนแก่น
*ผู้รับผิดชอบบทความ

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ORIGINAL ARTICLE

Determination of antibodies to crude *Opisthorchis viverrini* antigen insera of cholangiocarcinoma patients

Phongsaran Kimawaha[1,2,4], Apinya Jusakul[2,4], Paiboon Sithithaworn[3,4], Anchalee Techasen[2,4*]

Abstract

Over 8 million people in the northeastern region of Thailand are infected with liver flukes *Opisthorchis viverrini* (Ov), a cause of opisthorchiasis associated cholangiocarcinoma (CCA), a malignant tumor arising from bile duct epithelium. The proven oncogenic risk factor is Ov infection that can induce chronic inflammation and immune response. The present study aimed to investigate the level of serum IgG antibody to a crude Ov antigen in CCA patients, compared with a normal ultrasonography group by enzyme-linked immunosorbent assay (ELISA) and to study the correlations with clinicopathological parameters of CCA patients**.** Of 45 each for CCA and normal cases, 35.6% and 53.3% were positive for Ov IgG antibody, respectively. In addition, the level of IgG to Ov antigen was statistically associated with severity of tumor stage (p=0.036) and also had a trend to associate with shorter survival time of CCA patients. Statistical difference of IgG antibody level was not observed between CCA and normal ultrasonography group. In summary, antibody to crude Ov antigen in sera may not be used as a biomarker to predict the risk of CCA patients. The levels of Ov IgG antibody seem to be associated with the severity of CCA patients. However, molecular mechanisms in this issue are needed to be proven.

Keywords: *Opisthorchis viverrini*, Cholangiocarcinoma, Enzyme-linked immunosorbent assay, Arbitrary unit

[1]Biomedical Sciences, Graduate School, Khon Kaen University
[2]Faculty of Associated Medical Sciences, Khon Kaen University
[3]Department of Parasitology, Faculty of Medicine, Khon Kaen University
[4]Cholangiocarcinoma Research Institute, Khon Kaen University
*Corresponding author: (e-mail: anchte@kku.ac.th)

Introduction

Opisthorchiasis caused by liver flukes *Opisthorchis viverrini* (Ov), which is a foodborne trematode and infected over 8 million people in northeastern region of Thailand.[1] The traditional consumption of raw or uncooked fish contaminated metacercariae, the infective stage of Ov, is the major public health problem that contributes to humans becoming infected with this parasite.[2] It has been proved that Ov is an oncogenic risk factor associated with cholangio-carcinoma (CCA) development by chronic inflammation leading to oxidative stress and DNA damage.[3, 4] Additionally, CCA is a malignancy of bile duct epithelial cells arising from biliary tract. The pathophysiology of CCA is exceedingly invasive, it develops rapidly, regularly metastasizes, and is very difficult to diagnose until the disease reaches an advanced stage.[5] Consequently, the prevalence of Ov and CCA burden is strongly correlated and to eradicate this cancer, we have to find an effective way to prevent the incidence of Ov infection. The routine diagnosis of Ov in the laboratory is normally by detecting parasite eggs in feces as the "gold standard" and the intensity of infection is estimated as the number of Ov egg per gram (epg) of individual feces.[6] Fecal examination is easy, non-invasiveness and inexpensive but the sensitivity and specificity depends on the method of detection and the experience of personnel. In light infections or when egg output is low as a result of biliary obstruction or praziquantel treatment, anthelmintic drug, the fecal examination may result in a falsely negative diagnosis.[7] Furthermore, the characteristics of Ov eggs under light microscopy are difficult to distinguish from the eggs of minute intestinal flukes (MIFs) because of their small size that similar to Ov egg.[8] The limitation of the fecal examination in which diagnosis is based on egg morphology of parasites; it also emphasizes in term of the requirement for alternative and high-powered diagnostic methods in opisthorchiasis.

The development of Ov diagnosis based on serological tests is now widely studied with the goal to increase the sensitivity and specificity in a diagnostic assay. There are many acceptable tests for instance the intradermal test (IDT), immunoelectrophoresis (IEP), indirect haemagglutination assay (IHA), and enzyme-linked immunosorbent assay (ELISA).[9] For indirect ELISA method, it has been used to detect the specific antibody to Ov in patients' sera by using crude somatic extract or excretory secretory antigen (ES) which is the soluble metabolic products from adult worms.[10] The estimation of sensitivity for indirect ELISA is approximately 76-100% for IgG, 63% for IgA, and 74% for IgE.[11, 12] Therefore, the aim of this present study was to investigate the presence of serum IgG antibody to a crude Ov antigen in CCA patients compared with normal ultrasonography from a high endemic area and to correlate its level with clinicopathological data of CCA patients.

Materials and methods

Study subjects

Human CCA and normal sera in this study were approved by the Human Ethics Committee of Khon Kaen University, based on the ethics of human specimen experimentation of the National Research Council of Thailand (HE571283 and HE531320). Sera specimens were collected from CCA patients in Srinagarind hospital, Khon Kaen

(n=45) while a normal group, based on a radiologist examining by normal abdominal ultrasonography (n=45), comprises subjects who live in Ban Wa sub-distinct, Khon Kaen, Thailand where there is a high endemic area of Ov infection. These specimens were obtained from the biobank of the Cholangiocarcinoma Research Institute (CARI), Khon Kaen University. The Ov infected information in normal group was determined and quantified Ov EPG by microscopic fecal examination using the formalin-ethyl acetate concentration technique (FECT).[13]

Measurement of specific IgG antibody to crude Opisthorchis viverrini antigen by indirect enzyme-linked immunosorbent assay (Indirect-ELISA)

The indirect ELISA was performed to determine the levels of specific IgG antibody in serum. This assay was based on a crude extract of the antigens of whole adult Ov. The antigen concentration was diluted with 1xPBS pH 7.4 to give 1,500 µg/ml and distributed at 100 µl/well in a Maxisorp 96-well flat bottom microtiter plates and incubated at 4 ºC overnight. After that, plates were washed 3 times with 0.05% Tween20 in PBS pH 7.4 (PBST) and blocked with 250 µl of 3% skimmed milk powder in PBST and incubated at 37°C for 1 hour. Then, plates were decanted 5 times, followed by the addition of a test serum diluted in 3% skimmed milk in PBST (dilution 1:6000) at 100 µl/well in duplicates and incubated at 4°C overnight. After a further incubation time, plates were washed 5 times, and 100 µl of a conjugate of horseradish peroxidase with goat anti-human IgG was added to each well. After 2 hours' incubation at 37 ºC, the plate was washed and then a 100 µl of orthophenylenediamine hydrochloride (OPD)(Zymed, CA, USA) substrate was added for 30 minutes. The reaction was stopped with 4N sulfuric acid (H_2SO_4), and the plate was read on an ELISA reader (Tecan, Austria) using Magellan at the optical density (OD) of 492 nm. According to Jariwala et al.[14] assignment of arbitrary units (AUs) of antibody to the two-fold serial dilutions of human IgG against crude Ov antigen was performed to make the standard calibration curve by generating a four-parameter logistic log (4-PL) graph. The AUs of test sera were obtained by interpolating their ODs (492 nm) onto the 4-PL fitted standard calibration curve and then finding the corresponding values which are expressed as AUs. Any sample that gave AUs higher than the reliable detection limit (RDL), the concentration of antibody corresponding to the interpolated intersection of the lower asymptote of the upper 95% confidence limit with the lower 95% confidence limit of the standard data was considered positive for Ov infection.

Statistical analysis

Statistical analysis was performed using SPSS V.23.0 statistical software (IBM Corporation, USA). The descriptive statistics were used to analyze the association of antibody to crude Ov antigen in sera and clinicopathological parameters of CCA patients (e.g. sex, age, histological types, recurrence status, and tumor metastasis) between negative and positive results of antibody to crude Ov antigen. The log-rank test was used to compare survival distributions between negative and positive results of antibody and Kaplan–Meier method was plotted for survival curves for overall survival. The diagnostic performance of antibody to crude Ov antigen was evaluated using receiver operating characteristic (ROC) curve analysis, area

under the ROC curve (AUC) with 95% CI, Youden index, and Odds ratio were calculated and then the optimal cut-off AUs for antibody levels was designated to balance suitable sensitivity and specificity. Values of $P \leq 0.05$ were considered statistically significant.

Results

Characteristics of the study sample

The demographic data of the sample is shown in **Table 1**. The total number of study subjects was 90 cases including the normal (n=45) and CCA groups (n=45). The age range of all groups was 38 to 84 years and overall mean of age was 59.4 years (±SD = 10.3) being 57.7 years for males and 60.8 years for females. The Ov information as EPG was found to be positive in the normal ultrasonography group from endemic area in 10 cases (22.2%). Fecal specimens were not available for CCA patients.

Measurement of specific IgG antibody to crude Opisthorchis viverrini antigen by indirect-ELISA

Sera from 45 cases of CCA and 45 cases normal patients were included in this study. The assignment of arbitrary units (AUs) of antibody to the two-fold serial dilutions of human IgG against crude Ov antigen was performed to study. Using the AUs value of RDL that was 4.07 as the detection cut-off, from **Table 2**, positive for serum Ov IgG antibody of CCA patients was 16/45 (35.6%) and negative was 29/45 (64.4%). In addition, normal cases revealed positive and negative results for antibody to crude Ov antigen were 24 (53.3%) and 21 (46.7%) cases, respectively.

AUs levels of serum IgG to crude Ov antigen in negative and light Ov infection in normal ultrasonography group in endemic area

The status of Ov infection was examined by microscopic fecal examination (FECT). Normal individuals were categorized as to the intensity of Ov infection by the geometric of eggs per gram of feces as follows: “negative” or “0” (no eggs were found in feces) and “light infection” (1-499 eggs per gram of feces).[15] Levels of serum IgG to crude Ov antigen are shown in **Figure 1** between negative and light Ov infection in the normal group. The means of AUs levels were 6.57 ± 8.24 in negative and 8.62 ± 9.43 in light infection, respectively. There was no statistical difference in levels of antibody between negative and light Ov infection (P=0.51).

AUs levels of serum IgG to crude Ov antigen in normal and CCA patients

Figure 2 shows the serum level of IgG to Ov antigen, the means of AUs levels were 7.03 ± 8.54 in normal and 13.1 ± 35 in CCA, respectively. No statistical significant difference was found between groups (P=0.26).

Correlations between clinicopathological data and the specific IgG antibody to crude Ov antigen in CCA patients

The association between serum IgG to crude Ov antigen from indirect ELISA and clinicopathological variables of CCA patients was assessed. The results showed that positive of Ov IgG antibody in sera significantly correlated with TNM tumor stage 4 (P=0.036) of CCA patients. In different circumstances, no significant correlation of antibody to crude Ov antigen with age, gender,

histological types, recurrence, lymph node and distant metastatic stages was found as shown in **Table 3**.

Results of serum IgG to crude Ov antigen with survival rate of CCA patients

Figure 3 shows the overall survival (OS) analysis by the Kaplan-Meier method in which a log rank test revealed that there was a trend between positive serum Ov IgG antibody correlated with shorter survival time of CCA patients but it not reaches statistical significance (*P*=0.143). The mean overall survival times between negative and positive results of serum IgG to crude Ov antigen were 13.6 and 9.7 months, respectively.

Predictive values of serum IgG to crude Ov antigen for the detection of CCA patients compared to normal individuals

To evaluate the performance of serum IgG to crude Ov antigen as a potential biomarker for predicting opisthorchiasis associated with CCA, the receiver-operator characteristic (ROC) curve and area under curve (AUC) were analyzed. The AUs of serum IgG to crude Ov antigen based on the optimal cut-off derived from ROC analysis and Youden index calculation is presented in **Table 4**. ROC curve analysis showed that the serum IgG to crude Ov antigen level could not be used to differentiate between normal and CCA patients with AU cut-off was 2.48 at sensitivity 55.56% and specificity 66.67% (*P*=0.184, AUC=0.58, Youden index=121.23) and the ROC curve is shown in **Figure 4**. In addition, the odd ratio (OR) was determined to predict the risk of CCA relative to the normal group in cases of positive result of antibody to crude Ov antigen, and revealed that this elevated antibody had a trend to be a predictor to distinguish CCA from normal control group, but there was no statistical significance of odd ratio (OR=0.48, *P*=0.09).

Table 1 Demographic data of study subjects

Characteristics	Normal (%)	CCA (%)	Total (%)
Total (N)	45	45	90
Sex			
Male	12 (26.7)	29 (64.4)	41 (45.5)
Female	33 (73.3)	16 (35.6)	49 (54.5)
Age (years)			
30-39	0 (0)	2 (4.4)	2 (2.2)
40-49	8 (17.8)	3 (6.7)	11 (12.2)
50-59	16 (35.6)	18 (40)	34 (37.8)
60-69	10 (22.2)	16 (35.6)	26 (28.9)
70-79	9 (20)	5 (11.1)	14 (15.6)
80-89	2 (4.4)	1 (2.2)	3 (3.3)
Ov infection: Egg per gram (EPG)			
0	35 (77.8)	-	
1-499	10 (22.2)	-	

Table 2 Results of specific IgG antibodies to crude Ov antigens in sera of normal and CCA patients by assignment of arbitrary units (AUs)

Antibodies to crude Ov antigen	Normal	CCA	Total
	n=45 (%)	n=45 (%)	
Positive	24 (53.3)	16 (35.6)	40
Negative	21 (46.7)	29 (64.4)	50

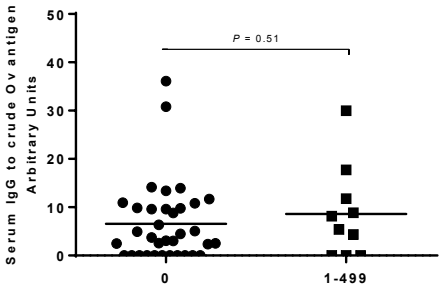

Figure 1 The serum levels of antibodies to crude Ov antigen depend on Ov infection status (EPG) in normal group. The EPG status "0" refers to negative infection and "1-499" refers to light infection. Black horizontal line refers to mean and *P*=0.51 that was no statistical significant.

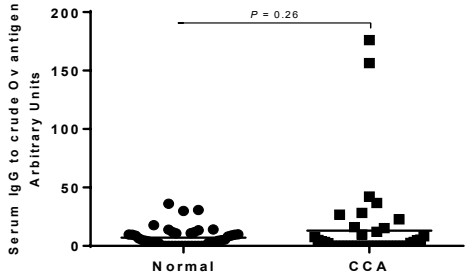

Figure 2 The serum levels of antibodies to crude Ov antigen in normal group and CCA patients. Black horizontal line refers to mean and *P*=0.26 that was no statistical significant.

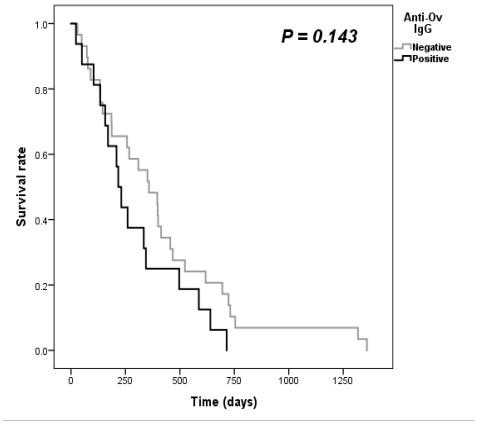

Figure 3 Overall survival curves of patients with CCA according to the specific IgG antibodies to crude Ov antigen results.

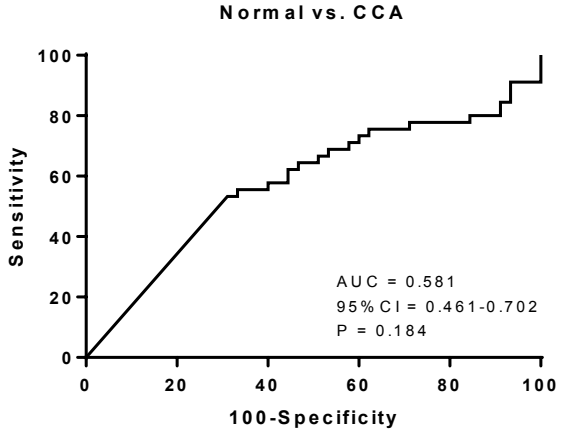

Figure 4 The receiver operator characteristic (ROC) curve to predict the performance of serum antibodies to crude Ov antigen in differentiate normal and CCA patients.

Table 3 Results of specific IgG antibodies to crude Ov antigens in sera of normal and CCA patients by assignment of arbitrary units (AUs)

Variables	No. of CCA patients	Antibodies to crude Ov antigen		*p* value
		Negative n=29 (%)	Positive n=16 (%)	
Age (Year)				
< 58.91	23	17 (58.6)	6 (37.5)	0.148
≥ 58.91	22	12 (41.4)	10 (62.5)	
Gender				
Male	29	20 (69)	9 (56.3)	0.297
Female	16	9 (31)	7 (43.7)	
Histological types				
Papillary	20	13 (44.8)	7 (43.7)	0.597
Non-Papillary	25	16 (55.2)	9 (56.3)	
Survival days				
< 368.22	27	15 (51.7)	12 (75)	0.113
≥ 368.22	18	14 (48.3)	4 (25)	
Recurrence				
Yes	18	11 (37.9)	7 (43.8)	0.472
No	27	18 (62.1)	9 (56.2)	
TNM stages				
I	2	1 (3.4)	1 (6.2)	**0.036***
IIA, IIB	4	4 (13.8)	0 (0)	
III	14	12 (41.4)	2 (12.5)	
IV	25	12 (41.4)	13 (81.3)	
Metastasis				
Yes	27	16 (55.2)	11 (68.8)	0.286
No	18	13 (44.8)	5 (31.2)	
Lymph node metastasis				
Yes	24	14 (48.3)	10 (62.5)	0.274
No	21	15 (51.7)	6 (37.5)	
Distant metastasis				
Yes	7	4 (13.8)	3 (18.7)	0.484
No	38	25 (86.2)	13 (81.3)	

Table 4 Predictive values of serum antibodies to crude OV antigen for the detection of CCA patients compared to normal individuals

Group comparisons	AUC (95% CI)	Cut-off (AUs)	Youden Index	Sensitivity (%)	Specificity (%)	*p* value	OR	*p* value
Normal vs. CCA	0.58 (0.46-0.70)	2.48	121.23	55.56	66.67	0.18	0.48	0.09

Discussion

This study shows that IgG antibody levels to crude Ov antigen may not predict the risk of CCA from normal ultrasonography because the sensitivity (55.6%) and specificity (66.7%) from ROC analysis are not sufficient to use this biomarker to predict CCA. In addition, the value of odd ratio (0.48) shows a rather low association to use this antibody in CCA diagnosis. Saichua et al.[15] also showed that serum antibodies to Ov antigen do not have the potential to predict CCA because of their lower sensitivity (54%), specificity (50%), and odd ratio (1.16) which was similar to our results. They did not found a statistical association between serum IgG to Ov antigen and intensity of Ov infection (0 egg and 1-499 eggs count) which support this current study. Furthermore, Saichua et al.[15] suggested that the levels of urine IgG to Ov antigen could serve as a biomarker to estimate the risk of CCA during Ov infection. We also first investigated the correlation of antibody to Ov antigen and clinicopathological parameters of CCA patients. Our results revealed that positive antibody to crude Ov antigen statistically correlated 81.3% (P=0.036) with cancer stage 4, which is a severe tumor stage. One possible interpretation of the close relationship reported between IgG levels and tumor stage is that the circulating antibodies to Ov antigens may occur from Ov adults in chronic infection and maybe influence severity in progression of CCA in the form of an immune complex that might be involved in CCA genesis. Niu N et al.[16] reported that IgG expression was associated with tumor differentiation, TNM stage, lymph node involvement and inflammatory infiltration in corolectal carcinoma. We also showed the trend that positive antibody to crude Ov antigen possibly associated with shorter survival rate of CCA patients. The mean overall survival times between negative and positive results of serum IgG to crude Ov antigen were 13.6 and 9.7 months, respectively.

Conclusion

This study found that antibody to crude Ov antigen in sera may not serve as biomarker to predict the risk of CCA patients from normal but it associated with severity of tumor stage in CCA patients. However, molecular mechanisms in this issue are needed to be proven.

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