



Helicobacter pylori GroEL Seropositivity Is Associated with an Increased Risk of *Opisthorchis viverrini*-Associated Hepatobiliary Abnormalities and Cholangiocarcinoma

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Abstract: Despite the synergistic effect of *Opisthorchis viverrini* and *Helicobacter pylori* co-infection on pathogenesis of severe hepatobiliary abnormalities (HBA) including advanced periductal fibrosis and replace with cholangiocarcinoma (CCA) have been established, the immune response to *H. pylori* in *O. viverrini* infected population has never been explored. Hence, this study aimed to investigate the antibody responses to 2 immunogenic *H. pylori* proteins in *O. viverrini*-infected patients with HBA and CCA. The risk analysis by multinomial logistic regression revealed that GroEL seropositivity was associated with higher risks of hepatobiliary abnormalities and CCA with adjusted odds ratios (95% confidence intervals) of 2.11 (95% CI = 1.20-3.71, $P=0.008$) and 2.13 (95% CI = 1.21-3.75, $P=0.009$), respectively. These findings indicate that GroEL seropositivity might be a biomarker for early detection of *O. viverrini* associated HBA and CCA.

Key words: *Opisthorchis viverrini*, *Helicobacter pylori*, risk factor, cholangiocarcinoma, hepatobiliary abnormality

INTRODUCTION

Infection with the liver fluke *Opisthorchis viverrini* is associated with the development of hepatobiliary abnormalities (HBA), including the bile duct cancer-cholangiocarcinoma (CCA) [1,2]. The incidence of CCA in the Northeast Thailand is the highest worldwide with 85 cases per 100,000 population [3]. CCA is associated with poor prognosis as most confirmed cases in the Northeast Thailand are stage IV cancers [4]. One major challenge is still early detection of the cancer as it would be associated with a better prognostic [5].

Among other factors, a carcinogenic bacterium, *Helicobacter pylori*, has been proposed to play an important role in carcinogenesis of CCA [6]. Previous studies demonstrated that *O. viverrini* is a reservoir host of *H. pylori* [7]. As a result, the preva-

lence of *H. pylori* infection was higher in *O. viverrini*-infected than uninfected populations [8]. Moreover, severe biliary periductal fibrosis during opisthorchiasis is associated with CagA-positive *H. pylori* infection [8]. Furthermore, co-infection of *O. viverrini* and *H. pylori* enhanced the severity of hepatobiliary abnormalities [9]. These findings support the hypothesis that *H. pylori* contributes to the pathogenesis of chronic opisthorchiasis and opisthorchiasis-associated CCA [10].

Antibody responses to *H. pylori* proteins were demonstrated to be associated with several cancers. For example, previous studies on gastric cancers showed that, among other proteins, seroreactivity to CagA and GroEL proteins of *H. pylori* was associated with cancer risk in a German cohort [11], a Swedish cohort [12], and East Asian cohorts [13]. In addition, seroreactivity to GroEL was associated with a higher risk of colorectal cancer in the US and Spain cohorts [14,15] and with a higher risk of hepatocellular carcinoma in a study on hepatobiliary cancers among Finnish people [16].

Despite the fact that the association between the 2 carcinogenic pathogens *O. viverrini* and *H. pylori* is now established, the immune response to *H. pylori* in *O. viverrini*-associated dis-

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eases has never been explored. Hence, this study focused on the detection of IgG antibodies to 2 *H. pylori* immunogenic antigens (CagA and GroEL). A case-control study was designed to determine the significance of antibody response in association with the risk of developing HBA and CCA in an *O. viverrini*-endemic area.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Institutional Human Ethics Committee of Khon Kaen University (HE 611280). Informed consent was obtained from each participant and patients enrolled in this study.

Study design and research subjects

This study was designed to analyse the IgG responses to 2 *H. pylori* antigens (virulent factor CagA and chaperonin GroEL) in serum samples collected from a community-based opisthorchiasis control in Northeast Thailand. Overall, 551 samples from patients aged between 30 and 74 years-old, were retrieved from the biobank of the Tropical Disease Research Center (TDRC, Academic Affairs, Faculty of Medicine, Khon Kaen University, Thailand). One hundred and twenty-two *O. viverrini*-negative people without hepatobiliary abnormalities were defined as controls and age- and sex-matched with 140 *O. viverrini*-positive patients without HBA, 144 *O. viverrini*-positive patients with HBA, and 145 patients with confirmed cholangiocarcinoma. Patients were selected among those who underwent stool examination by a quantitative formalin/ethyl acetate concentration technique for the evaluation of *Opisthorchis viverrini* infection. Among *O. viverrini*-positive patients, abdominal ultrasonography by a mobile high-resolution ultrasound machine was used to determine the presence of hepatobiliary abnormalities. These abnormalities included periportal fibrosis of the liver parenchyma, gallbladder wall and size, sludge and suspected cholangiocarcinoma (dilated bile duct and/or liver mass). Fibrosis grading is referring to periportal echoes in 2 to 3 segments or the liver (HBA+2) or more than 3 (HBA+3) [17]. Histologically confirmed CCA cases were selected among patients who underwent surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand.

Gene cloning and protein expression of partial *cagA* (*pcagA*) and *groEL*

H. pylori cagA and *groEL* genes were amplified from virulent strains isolated from Thai patients (provided by Professor Ratha-Korn Vilaichone, Thammasat University, Thailand). The details of gene cloning and protein expression of partial CagA (pCagA) and GroEL were described in the Supplementary Data S1 and Supplementary Table S1. Proteins were produced as soluble proteins of ~60-65 kDa for GroEL and pCagA (Supplementary Fig. S1).

pCagA and GroEL in-house indirect ELISA

After validation of positive and negative control sera with the commercial antibody determination kit E-Plate Eiken *H. pylori* antibody (Eiken Chemical Co., Ltd., Tokyo, Japan) and checkerboard titration of reagents, the following conditions were used. The microwell plates (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were coated with 0.05 µg proteins/well in sodium phosphate buffer (90 mM disodium phosphate, 130 mM monosodium phosphate, pH 6.5) overnight at 4°C. Wells were washed with 0.05% Tween 20 in PBS and blocked with 1% BSA in PBS for 2 hr. After washing, serum samples at 1:400 dilution in 1% BSA in PBS were added to individual wells in duplicate and incubated for 2 hr. After washing, a goat anti-human IgG-HRP (Thermo Fisher Scientific) was added at 1:60,000 in PBS for 1 hr. The wells were washed, and color reactions were developed with 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific). After 30 min incubation in the dark, reactions were stopped with 0.2 M sulphuric acid and plates read at 450 nm. Absorbance values (ODs) for all samples were calculated from the mean of 2 measurements and adjusted for interplate variation by dividing the ODs by the positive control OD of each corresponding experiment. The cut-off for positivity was calculated for each protein from the mean of the negative control ODs tested in all plates plus 3 SDs, to exclude positive outliers. Therefore, the negative threshold was 0.175 OD for pCagA ELISA experiments and 0.235 for GroEL experiments. ODs were corrected by subtracting each adjusted sample OD by the negative threshold. In addition, pCagA and GroEL serostatus were determined as follows: negative for corrected ODs equal to or less than 0; positive for all values strictly superior to 0.

Statistical analysis

All analyses were performed with RStudio software [18].

Correlation between anti-*H. pylori* antigens was analysed by Pearson's correlation (package stats, version 3.5.1, [19]). Results were plotted using raster package version 2.6-7 [20]. Correlations between intensity of *O. viverrini* infection (EPG levels) and pCagA and GroEL antibody levels were analysed by Kruskal-Wallis test (package stats, version 3.5.1, [19]). Excluding CCA patients for lack of data, the intensity of *O. viverrini* infection was assessed based on egg count (egg per gram of feces, EPG): OV negative for EPG=0; light infection for EPG<500; moderate for 500>EPG<1,000; and heavy for >1,000 EPG. Corrected sample ODs from pCagA and GroEL ELISA experiments were categorized in 5 groups: non-responders for OD=0 and low, moderate, high or very higher responders for OD>0 divided by quartiles. Results were plotted with ggplot2 package version 3.2.1 [21]. Risk analysis for *O. viverrini* infection, hepatobiliary abnormalities and cholangiocarcinoma was performed in association with GroEL and pCagA IgG serostatus by multinomial logistic regression (package nnet, version 7.3-12, [22]). Models were adjusted for age and sex and OV negative group was used as reference group.

RESULTS

Demographic data

Details of the tested population are listed in Supplementary Table S2. As samples were adjusted for age and sex among the groups, males constitute 53.3 to 55.2% of all samples in each group, and the mean age varied between 50.5- and 54.1-year-old. Among the 144 samples from OV+HBA+ patients, 52.1% presented HBA in 2 fragments of the liver while the remaining patients showed HBA in 3 fragments of the liver. Among CCA patients, the main represented cancers were well- (31%), moderately-differentiated adenocarcinomas (22.7%), and papillary adenocarcinomas (25.5%), followed by poorly differentiated adenocarcinomas (15.9%), adenosquamous carcinomas (3.5%) and squamous cell carcinomas (1.4%). Concerning cancer growth type, a mass-forming type (51%) was the most common type among patients. The growth type data were missing in 17.2% of all CCA patients.

Correlation between pCagA with GroEL IgG responses

Among all 551 samples, 19.2% were pCagA seronegative and 24.7% were GroEL seronegative. For pCagA, OD values of seropositive samples ranged from 0.0003 to 1.5 OD with a median of 0.2. For GroEL, OD ranged from 0.0023 to 1.8

(median OD: 0.3). Comparing pCagA with GroEL seropositive samples, a weak positive correlation was observed between pCagA and GroEL IgG responses ($r=0.19$, $P<0.01$, data not shown).

Correlation between pCagA and GroEL IgG responses with intensity of *O. viverrini* infection

Among 437 *O. viverrini* negative and positive samples with EPG data, EPG ranged from 0 to 2,439 with a median of 10 eggs per gram. Most samples (97.2%) showed light infections, with less than 500 EPG. In comparison with pCagA and GroEL IgG responses, no correlations were observed between EPG levels and pCagA IgG response ($P=0.49$, data not shown) and between EPG levels and GroEL IgG response ($P=0.076$, data not shown).

Association between seropositivity to *H. pylori* antigens and OV-associated HBA and CCA

Multinomial logistic regression, adjusted for age and sex, showed that GroEL seropositivity was a risk factor for hepatobiliary abnormalities and CCA with adjusted odds ratios (95% confidence intervals) of 2.11 (95% CI=1.20-3.71, $P=0.008$) and 2.13 (95% CI=1.21-3.75, $P=0.009$), respectively (Table 1). However, no such association was detected for pCagA.

DISCUSSION

The association between the 2 carcinogenic pathogens *O. viverrini* and *H. pylori* is now established. The presence of *H. pylori* genes is associated with *O. viverrini* infection and its associated pathologies [7-9]. In this study, we further investigated the antibody response to the virulent factor CagA and immunogenic GroEL proteins of *H. pylori* by ELISA. These proteins were selected based on previous studies that shown CagA was a well-known virulent factor and immunogenic protein of *H. pylori*, and GroEL has been shown as a potential independent risk factor for gastritis, gastric cancer and other cancers [11, 12,14,15]. Overall, the analysis showed that the IgG responses against CagA and GroEL were detected in all study groups. Besides, the response to these 2 antigens showed a weak positive yet significant correlation. These results indicated that the population was exposed to *H. pylori* infection.

Further analysis revealed that the level of antibodies to CagA protein and to GroEL showed no association to *O. viverrini* infection intensity. As more than 95% of the samples showed a

Table 1. Association between IgG responses to *Helicobacter pylori* antigens and *Opisthorchis viverrini*-associated HBA and CCA

	Control ^a n=122	OV+HBA- n=140	OV+HBA+ n=144	CCA n=145
pCagA seropositive	98 (80%)	112 (80%)	119 (85%)	117 (80%)
OR	1	1.03	1.22	1.07
95% CI	-	0.56-1.88	0.66-2.27	0.58-1.96
<i>P</i>	-	0.92	0.51	0.8
GroEL seropositive	82 (67%)	98 (70%)	117 (81%)	118 (81%)
OR	1	1.13	2.11	2.13
95% CI	-	0.67-1.92	1.20-3.71	1.21-3.75
<i>P</i>	-	0.62	0.009	0.008

Odds ratios, 95% confidence interval and *P*-value were calculated by multinomial logistic regression and significant *P*-values were noted in bold.

^aControl=reference group; OR=odds ratio; *P*=*P*-value; 95% CI=95% confidence interval; OV=*O. viverrini*; HBA=hepatobiliary abnormalities; CCA=cholangiocarcinoma.

light infection, this is a limitation of this study. It could be interesting to test more samples from moderately and heavily OV infected patients.

Next, we investigated whether the antibody response to *H. pylori* is associated with the pathological consequences of *O. viverrini*. In contrast to *O. viverrini* infection intensity, HBA and CCA were associated with antibody to GroEL; however, no such association was detected for CagA protein. GroEL is one of the most abundant proteins and expressed in most strains of *H. pylori* [23]. It was detected on the outside surface of the bacterium where it is likely exposed to the immune response [24]. Indeed, *H. pylori* GroEL was shown to activate NF- κ B and; thus, induce IL-8 production through TLR-2 and -4 associated pathways in gastric epithelial cells [25]. Pathogenesis of *O. viverrini*-associated hepatobiliary diseases are proposed as an inflammation induced process, and co-infection with *H. pylori* enhances the severity of *O. viverrini* infection [10]. Taken together, the immunogenic abundant GroEL might further enhance the inflammation and tissue damages, which in turn accelerate the carcinogenesis of *O. viverrini*-associated CCA.

Another interesting point of this study was that there was no association between CagA seropositivity and HBA. This finding contradicts results from a previous study showing that *cagA* gene is associated with HBA [8]. This discrepancy might be explained by gene expression variation of *cagA* gene due to the genetic heterogeneity of promoter region [26], gene motifs [27], and copy number [28]. These genetic variations might cause the differential gene expression of *cagA*, which in turn affects the immune response. However, this hypothesis warrants further investigation.

The limitation of this study was that we looked at the response to only 2 proteins of *H. pylori*. Several other proteins

have been involved in pathogenesis and are also biomarkers of *H. pylori* and its associated diseases [11]. We believed that employing a multiplex ELISA in a population-based study with a higher number of samples may provide a better understanding of the complex interaction between these 2 carcinogenic pathogens. Another issue that needs to be considered is that this study is not a longitudinal study. Therefore, it cannot answer whether *H. pylori* is the cause or the effect of HBA and CCA. Further studies are certainly needed to clarify this statement.

In conclusion, the results showed that GroEL seropositivity was a risk factor for both HBA and CCA. Further investigations regarding the potential use of GroEL as a biomarker for *O. viverrini*-associated diseases and the role of this protein in pathogenesis is needed, as the incidence of CCA in the northeast region of Thailand is still the highest worldwide.

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CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

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