Elevated Levels of Urinary 8-oxodG Correlate with Persistent Periductal Fibrosis after Praziquantel Treatment in Chronic Opisthorchiasis

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Abstract. Previous studies demonstrated that urinary 8-oxodG is a predictive biomarker for *Opisthorchis viverrini* (OV)-associated hepatobiliary disease (HBD) and cholangiocarcinoma (CCA). This study examined the effects of praziquantel treatment on the profile of urinary 8-oxodG in relation to HBD status. Infection with OV, levels of urinary 8-oxodG, and HBD status in terms of periductal fibrosis (PDF) assessed by abdominal ultrasonography (US) were monitored and compared in cohorts of participants in Khon Kaen, Thailand, before and 1 year after praziquantel treatment. Urinary 8-oxodG levels significantly decreased after treatment compared with the baseline level in OV-infected participants who had no HBD (PDF negative; PDF–ve) ($N = 14$). Levels of 8-oxodG were unchanged after treatment in OV-infected subjects (OV+ve) who had positive PDF ($N = 52$). Within the positive PDF (PDF+ve) group who became PDF–ve after treatment, there was no significant change in 8-oxodG levels between pre- and posttreatment (reversible PDF = 65.3%). In those who had persistent PDF+ve at both ultrasound sampling points, there was no significant difference in urinary 8-oxodG levels between pre- and posttreatment (persistent PDF = 34.6%). Based on a logistic regression model and receiver operation curve analysis, the increase of 8-oxodG levels was found to be associated with increasing risk of PDF. Measurement of urinary 8-oxodG and US increased the likelihood of discovering persistent PDF, which is a predictable condition for the patients at risk of OV-associated CCA. To identify high-risk individuals for CCA, it is useful to perform US in combination with urinary 8-oxodG measurement.

INTRODUCTION

The liver fluke, *Opisthorchis viverrini* (OV) is an important fish-borne trematode in Southeast Asia and has been recognized as a group I carcinogen in inducing hepatobiliary diseases (HBDs) and cholangiocarcinoma (CCA).^{1–3} Chronic inflammation from OV infection activates several oxidant-generating enzymes leading to oxidative stress and hence DNA damage.⁴ Oxidative stress causes DNA damage via modification of deoxyguanosine (8-oxodG, also known as 8-OHdG). DNA repairing system removes 8-oxodG formed in DNA and consequently the damaged nucleoside was excreted into urine.⁵ This DNA lesion is found in the nucleus of cancer cells in human CCA tissues and the prognosis is poor for the CCA patients who has a high level of 8-oxodG in their tumor tissues.^{4,6} Chronic opisthorchiasis also induces the overgrowth, hardening, and/or scarring of various tissues due to excess deposition of extracellular matrix components resulting in fibrosis⁷ and this eventually leads to cholangiocarcinogenesis.⁸ Opisthorchiasis and OV-associated CCA patients have elevated urinary 8-oxodG levels compared with healthy subjects, suggesting that it could be a biomarker for periductal fibrosis (PDF) and CCA, which is indicative of roles of oxidative stress in hepatobiliary pathology and cholangiocarcinogenesis.^{9,10}

Our community-based studies in northeastern Thailand revealed that removal of OV infection with praziquantel treatment reduced inflammation of HBD as measured by the

urinary 8-oxodG level.¹⁰ *Opisthorchis viverrini*-associated HBD mainly manifested as PDF and CCA.^{11–13} In abdominal ultrasonography (US), PDF is a major finding of OV-associated HBD, which is clinically a silent manifestation.^{12,14} However, the impact of praziquantel on the profile of urinary 8-oxodG in chronic opisthorchiasis with the presence of HBD, particularly PDF, have not yet been examined. Specifically, there is no information regarding the predictive value of urinary 8-oxodG after clearance of parasites with praziquantel treatment.

The aim of this study was to assess the impact of therapeutic outcomes of praziquantel treatment on HBD status (observed as PDF by US of OV-infected individuals) and to examine the profiles of urinary 8-oxodG to evaluate DNA damage due to hepatobiliary inflammation before and after praziquantel treatment. In addition, we evaluated urinary 8-oxodG levels as a predictive biomarker for PDF before and after praziquantel treatment in cohorts of participants.

METHODS

Study population and recruitment. The sample population was the residents in opisthorchiasis-endemic villages located in the Chi River wetland in Khon Kaen Province, northeastern Thailand. The study protocol was approved by the Ethics Committee of Khon Kaen University, Faculty School of Medicine, Khon Kaen, Thailand with the reference number HE581330. The target population recruited in this study comprised males and females with age > 20 years who had no evidence of alcohol-related liver disease or hepatic fibrosis, which was not related to opisthorchiasis as described previously.⁹

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After recruitment, demographic and life style data were obtained and all participants were screened for parasitic infections by fecal egg examination using the standard formalin–ethyl acetate concentration technique (FECT). Only those infected with OV were recruited for the study and the individuals who had eggs or larvae of other helminths were excluded. After sample collection and ultrasonographic examination, all OV-infected individuals were treated with praziquantel (40 mg/kg, single dose). The individuals were excluded from the study if they failed to provide complete urine and feces specimens at baseline (before) and after treatment with praziquantel. The participants were further excluded if their urine specimens were suspected of having urinary tract infection or other comorbidities after the urine strip test (Supplemental Figure 1).

The participants finally enrolled in this study were divided into two groups by the status of parasite infection and HBD. Group 1 ($N = 23$) consisted of healthy individuals who had no parasitic infection and were negative for PDF (PDF–ve) after abdominal US. Group 2 ($N = 66$) consisted of individuals who were infected with OV and were followed up for 1 year after praziquantel treatment. The individuals in Group 2 had received ultrasound examinations at pre- and 1 year posttreatment and were then divided further into two subgroups according to HBD status as PDF–ve and PDF positive (PDF+ve) (Supplemental Figure 1).

Clinical specimens. Feces and urine specimens were collected from each participant on the same day at pre- and posttreatment. The stool samples were stored in chilled insulated box (12–15°C), transported to our laboratory, and fixed with formalin on the same day until processing for fecal examination. The morning, mid-stream urine samples were collected into clean plastic containers and were kept on ice in an insulated box. The specimens were then transported to the laboratory within the same day.

Fecal examination. The quantitative FECT was performed for parasitological screening of OV and other parasitic infection as previously described.¹⁵ The results were obtained from processing of 2 g of fecal samples, and the final sediment was examined twice for the parasite eggs and were recorded to calculate prevalence and intensity of infection (eggs/g feces) at pre- and post-praziquantel treatment. Morphological identification between OV and minute intestinal flukes (MIFs) was carefully considered as described in the previous reports,^{15,16} and this study was carried out in Khon Kaen, known as the endemic area of OV with low prevalence of MIFs.¹⁷

Urinalysis and 8-oxodG measurement. The urine specimens were first screened for urinary tract infection using the urine strip test (AUTION sticks; ARKRAY, Kyoto, Japan) read and analyzed on an automated urine chemistry analyzer (AUTION MAX AX-4280; ARKRAY America, Minneapolis, MN). Specimens that showed abnormal values were excluded from the study, that is, leukocyte or nitrate positive. The urine specimens with normal values were centrifuged and the supernatant was kept at –20°C for further analysis.

The levels of 8-oxodG (ng/mL) in urine were measured using a validated competitive HT 8-oxodG ELISA kit (Trevigen, Gaithersburg, MD) as previously described.⁹ Optical density at 450 nm was determined using SpectraMax 340PC384 system, (Molecular Devices, Sunnyvale, CA). The SoftMax Pro Software (Molecular Devices) was used for data management. The standard curve was created using a polynomial function and the 8-oxodG levels in urine were interpolated onto this standard curve and expressed as nanograms per milliliter.

Normalization of 8-oxodG using specific gravity (SG) of urine. The SG of each unprocessed urine samples was determined using a urine strip test (AUTION sticks; ARKRAY) analyzed on an AUTION MAX AX-4280 automated urine

TABLE 1

Median and interquartile range (25th–75th percentile) of urinary 8-oxodG levels adjusted with specific gravity (ng/mL) in individuals infected with OV at pretreatment and 1 year post-praziquantel treatment

Characteristics	N	Pretreatment	Posttreatment	P value*
				Pre vs. post
Gender				
Male	41	162.54 (119.65–222.16)	157.37 (123.42–239.14)	0.78
Female	25	161.58 (110.84–217.46)	141.60 (109.48–234.03)	0.40
Age				
30–39	13	229.15 (170.33–302.90)	167.59 (125.41–232.54)	0.03
40–49	18	125.80 (103.67–178.89)	156.28 (136.88–231.21)	0.06
50+	35	161.58 (110.45–215.17)	141.60 (111.25–216.07)	0.35
Smoking status				
No	27	163.47 (114.45–199.24)	141.60 (111.25–216.07)	0.37
Yes	38	159.58 (113.98–218.67)	159.41 (122.83–240.31)	0.94
Alcohol consumption				
No	9	161.58 (71.30–187.55)	131.20 (107.27–182.41)	0.85
Yes	56	162.35 (119.26–225.66)	157.14 (123.17–233.12)	0.62
Intensity† of OV infection (EPG)				
1–499	54	162.35 (117.02–203.20)	154.96 (119.96–228.08)	0.64
500–999	8	235.15 (111.45–313.01)	160.33 (105.28–238.89)	0.20
≥ 1,000	4	122.96 (97.83–233.60)	175.77 (125.20–265.43)	0.14
Curative after treatment				
Cured	63	162.17 (114.45–215.08)	154.25 (119.62–231.16)	0.66
Not-cured	3	236.68 (125.58–NA)	156.91 (132.83–NA)	0.28

EPG = eggs per gram of feces; NA = data not available; OV = *Opisthorchis viverrini*; Post = post 1 year treatment with praziquantel; Pre = pretreatment with praziquantel.

* Wilcoxon matched pairs test was used to determine probability of 8-oxodG levels before and after praziquantel treatment.

† Intensities of OV infection are categorized by the geometric mean of EPG.

FIGURE 1. Comparison of urinary 8-oxodG levels between groups of participants who were PDF+ve, PDF-ve, and healthy before treatment. PDF-ve = periductal fibrosis negative; PDF+ve = periductal fibrosis positive.

chemistry analyzer (ARKRAY America). To standardize the 8-oxodG levels in urine, the adjustment was made using the following formula where SG_{avg} is the average of SG in all samples and SG is the SG in the sample; SG-corrected concentration (ng/mL) = (8-oxodG concentration ng/mL) $\times$ ($SG_{avg} - 1 / SG - 1$). This formula has been previously used to correct urinary tobacco exposure biomarkers, urinary cadmium, urinary arsenic, 1,6-hexamethylene diamine, urinary protein and albumin, and other toxic substances in urine.¹⁸ In cases of missing data of SG in some individuals at baseline or posttreatment, the 8-oxodG levels were adjusted using the average SG from the overall urine samples.

Ultrasonographic detection of PDF. A mobile, high-resolution US machine was used for abdominal ultrasound screening for hepatobiliary abnormalities in opisthorchiasis. In this study, ultrasonographic hepatobiliary abnormalities were graded and recorded according to the criteria described in our previous reports.^{14,19} Individuals were classified as “normal or non-PDF” (PDF-ve) when the US showed normal echo pattern of the liver parenchyma. Individuals were classified as “PDF” (PDF+ve) grade 1–3 if the US showed increased periportal echo. The ultrasonographic observation was performed twice, at baseline (before) and 1 year after praziquantel treatment.

Praziquantel administration. After collection of the baseline specimens and ultrasound examination, the OV-infected participants were treated with a standard dose of praziquantel (40 mg/kg body weight). These cohorts of participants were then followed up at 1 year after treatment.

Statistical analysis. Statistical analyses were conducted using SPSS version 22 (IBM, Chicago, IL). Descriptive statistics, including the number of observations (*N*), median, and interquartile range (IQR), were calculated for demographic data. The normality of data distribution was assessed using the Kolmogorov–Smirnov test. The Kruskal–Wallis analysis of variance was used to test the differences of urine 8-oxodG levels between demographic variables of the project participants.

At 1 year posttreatment, abdominal US examination of the participants who were originally PDF+ve were classified into two groups, namely, the reversible PDF group (from PDF+ve to PDF-ve at pre- and posttreatment) and the persistent PDF group (PDF+ve at pre- and posttreatment). The differences of urine 8-oxodG levels between pre- and posttreatment in each subgroup were validated with the Wilcoxon matched pairs test. The 8-oxodG levels at pre- and posttreatment of PDF+ve or PDF-ve in comparison with endemic normal were determined using the Mann–Whitney *U* test.

The receiver operator characteristic (ROC) curves of 8-oxodG levels at baseline and posttreatment were constructed using the MedCalc statistical software 11 (MedCalc Software, Ostend, Belgium) and VassarStats (Vassar College, Poughkeepsie, NY, USA). Sensitivity, specificity, positive predictive value, and negative predictive value for each cutoff to predictor in PDF+ve were calculated. Predictive values of urine 8-oxodG as the risk for PDF were estimated based on the logistic regression model using univariate analyses as well as multivariate for age, gender, smoking, and alcoholic drink as covariates.

RESULTS

Descriptive statistics for urinary 8-oxodG in the participants. The urinary 8-oxodG levels in healthy controls showed no difference between categories of demographic and life style data (Supplemental Table 1). Among opisthorchiasis participants whose age was less than 40 years, there was a significant decrease in 8-oxodG levels after praziquantel treatment (Table 1).

Urinary 8-oxodG levels in relation to HBD status. The urinary 8-oxodG level (median and IQR) at the baseline of OV-infected individuals who were PDF+ve (167.10,

FIGURE 2. Comparison of urinary 8-oxodG levels at pre- and post-praziquantel treatment (A) PDF-ve (*N* = 14) and (B) PDF+ve (*N* = 52). Wilcoxon matched pairs test; **P* < 0.05. PDF-ve = periductal fibrosis negative; PDF+ve = periductal fibrosis positive.

FIGURE 3. Comparison of urinary 8-oxodG levels in reversal and persistent periductal fibrosis (A) PDF+ve individuals who became PDF-ve after treatment ($N = 34$) (B) PDF+ve/PDF+ve ($N = 18$). PDF-ve = periductal fibrosis negative; PDF+ve = periductal fibrosis positive.

115.78–236.43) was higher than those who were PDF-ve (141.29, 109.64–185.17), but the difference was not statistically significant (Figure 1). The urinary 8-oxodG levels in both groups were significantly higher than that of the healthy subject (62.23, 51.40–96.24; $P < 0.001$).

Effects of praziquantel treatment. One year after praziquantel treatment, most OV-infected individuals became egg-negative (63/66 = 95.45%), whereas three individuals still had OV infection (3/66 = 4.55%) as determined by fecal examination. Of the three OV-infected individuals who were PDF+ve at pretreatment, one became PDF-ve and the other two remained PDF+ve after treatment (Supplemental Figure 1).

The 8-oxodG level (median and IQR) of OV-infected individuals who were PDF-ve at 1 year after treatment was significantly lower than that of those at baseline (pretreatment) (117.25, 102.53–148.16 versus 141.29, 109.64–185.17; $P < 0.05$, $N = 14$) (Figure 2A). By contrast, in OV-infected individuals who were PDF+ve ($N = 52$), there was no significant difference of urinary 8-oxodG levels before and after praziquantel treatment (Figure 2B).

Further subgrouping of the PDF+ve and OV+ve group ($N = 52$) based on hepatobiliary pathology outcome at posttreatment showed that there was no significant change in the urinary 8-oxodG between pre- and posttreatment in individuals who were initially PDF+ve at baseline and who became PDF-ve after praziquantel treatment (the reversible group, $N = 34$) (Figure 3A). Likewise, the remaining individuals who were PDF+ve at pre- and 1 year posttreatment (the persistent PDF group, $N = 18$) did not show significant changes in the urinary 8-oxodG levels before and after praziquantel treatment (Figure 3B).

Urinary 8-oxodG as a risk biomarker for PDF. The ROC curve in Supplemental Figure 2A and B showed that the predictive power of urinary 8-oxodG in identifying PDF was 75.2% and 62.6% for pre- and posttreatment, respectively. The optimal diagnostic threshold of urinary 8-oxodG at pretreatment and posttreatment conditions to identify PDF+ve

status was 71.91 and 155.66 ng/mL, respectively. The efficiency of urinary 8-oxodG for diagnosis of OV-associated PDF before and 1 year after treatment according to sensitivity and specificity are shown in Table 2.

The logistic regression model was used to determine the odds of having PDF (PDF+ve) in relation to the urinary 8-oxodG levels at pre- and posttreatment and the results are presented in Table 3. There was an increasing trend of odds ratios with the increase of urinary 8-oxodG levels at both pre- and posttreatment, but a statistically significant correlation was observed only at pretreatment ($P < 0.05$). Age, gender, smoking, and alcoholic drinks were included in the model to avoid these confounding factors. However, those factors do not have significant effects on the risk of PDF (data not shown).

DISCUSSION

The significant reduction in the urinary 8-oxodG level at 1 year after praziquantel treatment in OV-infected individuals without PDF (PDF-ve) observed in this study was in concordance with our previous report.¹⁰ By contrast, the urinary 8-oxodG level in the OV-infected PDF+ve subjects did not change 1 year after praziquantel treatment. Because the urinary 8-oxodG level represents inflammatory reactions of the body in total, the reduction observed after parasite removal suggests a significant contribution of OV-related hepatobiliary inflammation to the urinary 8-oxodG level. In the present study, the urinary 8-oxodG level was significantly reduced only in the PDF-ve group, but such a change was not seen in the PDF+ve group, indicating the contribution of duration or stage of OV infection on the oxidative stress. It has been postulated that the hepatobiliary inflammation is reversible in the early phase of OV infection but not in the chronic phase of infection.^{8,20}

The data that there was no change in 8-oxodG levels after worm removal by praziquantel treatment in the PDF+ve group

TABLE 2
Performance of urinary 8-oxodG at pre- and 1 year posttreatment of prediction of *Opisthorchis viverrini*-associated PDF

	Sensitivity (95% CI)	Specificity (95% CI)	Predictive value (%) (95% CI)		Likelihood ratio	
			Positive	Negative	Positive	Negative
PDF pretreatment	96.15 (86.8–99.5)	43.24 (27.1–60.5)	69.4 (57.3–79.5)	88.2 (62.3–97.9)	1.69	0.089
PDF posttreatment	72.22 (46.5–90.3)	60.42 (45.3–74.2)	39.4 (23.4–57.8)	84.8 (67.3–94.3)	1.82	0.460

CI = confidence interval; PDF = positive for periductal fibrosis as determined by abdominal ultrasonography.

TABLE 3
Predictive risk values (odd ratios) for periductal fibrosis classified by urinary 8-oxodG levels at pre- and posttreatment group

Range of 8-oxodG* (ng/mL)	Pretreatment		Range of 8-oxodG (ng/mL)	Posttreatment	
	aOR	95% CI		aOR	95% CI
0–90.22	1.000	–	0–119.96	1.000	–
90.23–141.29	3.235	0.757–13.817	119.97–154.96	3.407	0.315–36.905
141.30–193.66	6.242	1.179–33.049	154.97–228.08	12.901	1.272–130.812
> 193.66	9.006	1.878–43.202	> 228.08	4.940	0.475–51.380

aOR = adjusted odds ratios by age and gender; CI = confidence interval.

* Urinary 8-oxodG levels were adjusted with specific gravity.

imply that the persistency of PDF may be driven by several mechanisms. Currently, there are at least three possibilities. First possibility is due to OV-induced inflammation because OV antigen has been demonstrated in bile duct epithelial cells, periportal hepatocyte, and fibrotic tissue²¹ and also the detection of excretory–secretory OV antigen in urine in infected individuals.²² Similar findings for the presence of parasite antigen in the body tissue have been reported in *Schistosoma haematobium*–associated urothelial carcinoma inflammation and cancer tissues.²³ Second, persistent biliary fibrosis may be driven by a pro-inflammatory cytokine, interleukin-6 (IL-6), released from the fibrotic tissue.²⁴ A previous study revealed that the level of IL-6 was eight times higher in human opisthorchiasis with advanced PDF than those without advanced PDF.¹⁹ In schistosomiasis, IL-13 was found to contribute to the persistency of fibrosis after praziquantel treatment.²⁵ Third, OV was found to act as a reservoir for *Helicobacter pylori* in the OV-infected individuals and coinfection with this bacteria may support the pathogenesis of liver fluke-induced HBDs, including CCA.²⁶ Further evidence is required to prove or disprove the likelihood of those three possibilities.

It is known that life style factors such as smoking and alcoholic consumption are associated with oxidative stress and 8-oxodG level.²⁷ With the assumption that there was no change in life style pattern in the cohort of participants in our study, persistent inflammation related to persistent PDF occurred in 34% of the subjects, which are considered at high risk for CCA. Because treatment by praziquantel has been widely used for parasite control in opisthorchiasis, it also reduces bile duct inflammation.²⁸ Praziquantel treatment was reported to reverse the status of HBD in an OV-endemic community²⁹ and eventually reduced the risk for CCA development. In our study, the OV-infected individuals were treated with a single dose of 40 mg/kg praziquantel and the cure rate assessed at 1 year posttreatment was 95.45% (63/66). It has been reported that an increased dose of praziquantel to 75 mg/kg (3 × 25 mg/kg) was more efficacious than 40 mg/kg.^{30–32} Obviously, further studies are required to determine whether administering a higher dose of praziquantel improves the cure rate and reduces bile duct inflammation.

Currently, there are limitations in using urinary 8-oxodG level as a disease biomarker in screening for high-risk individuals for CCA. In this study, to compensate variation in the volume of urine, urinary 8-oxodG levels were adjusted using urine SG. Specific gravity is a potentially useful alternative to urinary creatinine (U-Cre) to correct and normalize urinary chemical data and determination of biomarkers.³³ The creatinine adjustment of spot urine concentrations is based on the constant rate of creatinine excretion in human urine. However, U-Cre varies by age, gender, body size, and diet.^{34,35}

When adjusting different solutes in urine for U-Cre, those factors will affect the obtained concentrations. Moreover, U-Cre levels also differed significantly across disease groups in cystic fibrosis.³⁶ The use of SG was recommended to adjust urinary 8-oxodG as disease biomarkers in epidemiological studies.³⁷

Several studies have used US to identify individuals with PDF as a risk group for CCA development. In this study, the protocol and criterion of ultrasound diagnosis was conducted by expert radiologists, and validation of results were performed by the teleconsultation US platform.^{12,38} The sensitivity of ultrasound diagnosis in suspected acute biliary tract disease was reported to be 83% compared with computed tomography scan and 99% for detecting ductal dilatation compared with endoscopic retrograde cholangiopancreatography.³⁹ Based on the findings that urinary 8-oxodG level is more sensitive than US for evaluation of hepatobiliary inflammation, we propose that a combination of urinary 8-oxodG⁴⁰ and urine antigen assays for opisthorchiasis^{9,22} should be used in the screening strategy for PDF and early CCA. This screening strategy can be added to the current screening protocol for CCA⁴¹ in that specific inclusion criteria are age > 40, history of OV infection, family history of liver cancer, and native of Isan. As such, it is anticipated that a higher percentage of PDF+ve including persistent PDF will be uncovered.

This study has a number of limitations, the most serious of which is the small sample size because of constraints and logistics as a part of a larger project started in 2012 with an initial cohort of 1,477.⁹ Further limitations were the lack of sufficient number of available samples at base line, which are needed to be from the same individual(s) at 1-year follow-up and also sufficient subjects who provide matched pair of stool and urine samples. Furthermore, number of recruited subjects decreased because they were excluded according to concurrent parasitic infection (26%) and comorbidity of urinary tract infection from the urine strip tests (30%) and finally was the filed coordination because the sample subjects were distributed in 13 different villages in Khon Kaen.

In conclusion, the results of this study showed that praziquantel treatment in opisthorchiasis contributed to reduce oxidative DNA damage measured by urinary 8-oxodG in HBDs of both acute (reversal PDF) and chronic biliary fibrosis (persistent PDF). Within the group of PDF+ve at baseline screening, more than half of the participants (65.38%) showed reversal of PDF at 1 year posttreatment. The proportion of the subjects with persistent HBD (PDF+ve) at pre- and posttreatment accounted for one-third of the participants (34.62%). Repeated ultrasound screening for the persistent PDF+ve individuals is essential to discover emerging early CCA

cases for curative treatment.⁴¹ In this study, more than 34% of persistent PDF after praziquantel treatment was associated with high urinary 8-oxodG level, suggesting that more risk group of CCA could be identified if urinary 8-oxodG were used in combination with current screening for PDF by US. The ease and noninvasiveness of sample collection has made urinary 8-oxodG suitable for initial screening in the field at a point-of-care test setting in the endemic areas, and it is an attractive approach for screening hepatobiliary pathologies.

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REFERENCES

- Elkins DB, Haswell-Elkins MR, Mairiang E, Mairiang P, Sithithaworn P, Kaewkes S, Bhudhisawasdi V, Uttaravichien T, 1990. A high frequency of hepatobiliary disease and suspected cholangiocarcinoma associated with heavy *Opisthorchis viverrini* infection in a small community in north-east Thailand. *Trans R Soc Trop Med Hyg* 84: 715–719.
- IARC, 1994. Infection with liver flukes (*Opisthorchis viverrini*, *Opisthorchis felinus* and *Clonorchis sinensis*). *IARC Monogr Eval Carcinog Risks Hum* 61: 121–175.
- Mairiang E, Elkins DB, Mairiang P, Chaikyakum J, Chamadol N, Loapaiboon V, Posri S, Sithithaworn P, Haswell-Elkins M, 1992. Relationship between intensity of *Opisthorchis viverrini* infection and hepatobiliary disease detected by ultrasonography. *J Gastroenterol Hepatol* 7: 17–21.
- Thanan R, Oikawa S, Hiraku Y, Ohnishi S, Ma N, Pinlaor S, Yongvanit P, Kawanishi S, Murata M, 2015. Oxidative stress and its significant roles in neurodegenerative diseases and cancer. *Int J Mol Sci* 16: 193–217.
- Evans MD, Saparbaev M, Cooke MS, 2010. DNA repair and the origins of urinary oxidized 2'-deoxyribonucleosides. *Mutagenesis* 25: 433–442.
- Murata M, Thanan R, Ma N, Kawanishi S, 2012. Role of nitrate and oxidative DNA damage in inflammation-related carcinogenesis. *J Biomed Biotechnol* 2012: 623019.
- Wynn TA, 2008. Cellular and molecular mechanisms of fibrosis. *J Pathol* 214: 199–210.
- Yongvanit P, Pinlaor S, Bartsch H, 2012. Oxidative and nitrate DNA damage: key events in opisthorchiasis-induced carcinogenesis. *Parasitol Int* 61: 130–135.
- Saichua P et al., 2015. Levels of 8-OxodG predict hepatobiliary pathology in *Opisthorchis viverrini* endemic settings in Thailand. *PLoS Negl Trop Dis* 9: e0003949.
- Thanan R, Murata M, Pinlaor S, Sithithaworn P, Khuntikeo N, Tangkanakul W, Hiraku Y, Oikawa S, Yongvanit P, Kawanishi S, 2008. Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine in patients with parasite infection and effect of antiparasitic drug in relation to cholangiocarcinogenesis. *Cancer Epidemiol Biomarkers Prev* 17: 518–524.
- Elkins DB, Mairiang E, Sithithaworn P, Mairiang P, Chaikyakum J, Chamadol N, Loapaiboon V, Haswell-Elkins MR, 1996. Cross-sectional patterns of hepatobiliary abnormalities and possible precursor conditions of cholangiocarcinoma associated with *Opisthorchis viverrini* infection in humans. *Am J Trop Med Hyg* 55: 295–301.
- Mairiang E, Laha T, Bethony JM, Thinkhamrop B, Kaewkes S, Sithithaworn P, Tesana S, Loukas A, Brindley PJ, Sripan B, 2012. Ultrasonography assessment of hepatobiliary abnormalities in 3359 subjects with *Opisthorchis viverrini* infection in endemic areas of Thailand. *Parasitol Int* 61: 208–211.
- Mairiang E, Mairiang P, 2003. Clinical manifestation of opisthorchiasis and treatment. *Acta Trop* 88: 221–227.
- Chamadol N, Pairojkul C, Khuntikeo N, Loapaiboon V, Loilome W, Sithithaworn P, Yongvanit P, 2014. Histological confirmation of periductal fibrosis from ultrasound diagnosis in cholangiocarcinoma patients. *J Hepatobiliary Pancreat Sci* 21: 316–322.
- Kaewkes S, Elkins DB, Sithithaworn P, Haswell-Elkins MR, 1991. Comparative studies on the morphology of the eggs of *Opisthorchis viverrini* and leicithodendriid trematodes. *Southeast Asian J Trop Med Public Health* 22: 623–630.
- Tesana S, Srisawangwonk T, Kaewkes S, Sithithaworn P, Kanla P, Arunyanart C, 1991. Eggshell morphology of the small eggs of human trematodes in Thailand. *Southeast Asian J Trop Med Public Health* 22: 631–636.
- Songserm N, Promthet S, Wiangnon S, Sithithaworn P, 2012. Prevalence and co-infection of intestinal parasites among Thai rural residents at high-risk of developing cholangiocarcinoma: a cross-sectional study in a prospective cohort study. *Asian Pac J Cancer Prev* 13: 6175–6179.
- Xia Y, Wong LY, Bunker BC, Bernert JT, 2014. Comparison of creatinine and specific gravity for hydration corrections on measurement of the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine. *J Clin Lab Anal* 28: 353–363.
- Sripan B, Mairiang E, Thinkhamrop B, Laha T, Kaewkes S, Sithithaworn P, Tesana S, Loukas A, Brindley PJ, Bethony JM, 2009. Advanced periductal fibrosis from infection with the carcinogenic human liver fluke *Opisthorchis viverrini* correlates with elevated levels of interleukin-6. *Hepatology* 50: 1273–1281.
- Sripan B, Brindley PJ, Mulvanna J, Laha T, Smout MJ, Mairiang E, Bethony JM, Loukas A, 2012. The tumorigenic liver fluke *Opisthorchis viverrini*—multiple pathways to cancer. *Trends Parasitol* 28: 395–407.
- Sripan B, Kaewkes S, 2000. Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. *Int J Parasitol* 30: 735–740.
- Worasith C et al., 2015. Advances in the diagnosis of human opisthorchiasis: development of *Opisthorchis viverrini* antigen detection in urine. *PLoS Negl Trop Dis* 9: e0004157.
- Wishahi M, Zakarya A, Hamamm O, Abdel-Rasol M, Badawy H, Elganzoury H, Ismail M, Elkhoully A, Meheina A, 2014. Impact

- of density of schistosomal antigen expression in urinary bladder tissue on the stratification, cell type, and staging, and prognosis of carcinoma of the bladder in Egyptian patients. *Infect Agent Cancer* 9: 21.
24. Sundararaj KP, Samuvel DJ, Li Y, Sanders JJ, Lopes-Virella MF, Huang Y, 2009. Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: cross-talking between fibroblasts and U937 macrophages exposed to high glucose. *J Biol Chem* 284: 13714–13724.
 25. Martins-Leite P, Gazzinelli G, Alves-Oliveira LF, Gazzinelli A, Malaquias LC, Correa-Oliveira R, Teixeira-Carvalho A, Silveira AM, 2008. Effect of chemotherapy with praziquantel on the production of cytokines and morbidity associated with schistosomiasis mansoni. *Antimicrob Agents Chemother* 52: 2780–2786.
 26. Deenonpoe R, Chomvarin C, Pairojkul C, Chamgramol Y, Loukas A, Brindley PJ, Sripa B, 2015. The carcinogenic liver fluke *Opisthorchis viverrini* is a reservoir for species of *Helicobacter*. *Asian Pac J Cancer Prev* 16: 1751–1758.
 27. Loft S, Vistisen K, Ewertz M, Tjonneland A, Overvad K, Poulsen HE, 1992. Oxidative DNA damage estimated by 8-hydroxydeoxyguanosine excretion in humans: influence of smoking, gender and body mass index. *Carcinogenesis* 13: 2241–2247.
 28. Pinlaor S et al., 2006. iNOS-dependent DNA damage via NF-kappaB expression in hamsters infected with *Opisthorchis viverrini* and its suppression by the antihelminthic drug praziquantel. *Int J Cancer* 119: 1067–1072.
 29. Mairiang E, Haswell-Elkins MR, Mairiang P, Sithithaworn P, Elkins DB, 1993. Reversal of biliary tract abnormalities associated with *Opisthorchis viverrini* infection following praziquantel treatment. *Trans R Soc Trop Med Hyg* 87: 194–197.
 30. Chai JY, 2013. Praziquantel treatment in trematode and cestode infections: an update. *Infect Chemother* 45: 32–43.
 31. Lovis L et al., 2012. Efficacy of praziquantel against *Schistosoma mekongi* and *Opisthorchis viverrini*: a randomized, single-blinded dose-comparison trial. *PLoS Negl Trop Dis* 6: e1726.
 32. Saengsawang P, Promthet S, Bradshaw P, 2013. Infection with *Opisthorchis viverrini* and use of praziquantel among a working-age population in northeast Thailand. *Asian Pac J Cancer Prev* 14: 2963–2966.
 33. Miller RC, Brindle E, Holman DJ, Shofer J, Klein NA, Soules MR, O'Connor KA, 2004. Comparison of specific gravity and creatinine for normalizing urinary reproductive hormone concentrations. *Clin Chem* 50: 924–932.
 34. Hall Moran V, Leathard HL, Coley J, 2001. Urinary hormone levels during the natural menstrual cycle: the effect of age. *J Endocrinol* 170: 157–164.
 35. Worsfold M, Davie MW, Haddaway MJ, 1999. Age-related changes in body composition, hydroxyproline, and creatinine excretion in normal women. *Calcif Tissue Int* 64: 40–44.
 36. Wagner BD, Accurso FJ, Laguna TA, 2010. The applicability of urinary creatinine as a method of specimen normalization in the cystic fibrosis population. *J Cyst Fibros* 9: 212–216.
 37. Suwazono Y, Akesson A, Alfven T, Jarup L, Vahter M, 2005. Creatinine versus specific gravity-adjusted urinary cadmium concentrations. *Biomarkers* 10: 117–126.
 38. Chamadol N, Laopaiboon V, Srinakarin J, Loilome W, Yongvanit P, Thinkhamrop B, Khuntikeo N, 2017. Teleconsultation ultrasonography: a new weapon to combat cholangiocarcinoma. *ESMO Open* 2: e000231.
 39. O'Connor OJ, O'Neill S, Maher MM, 2011. Imaging of biliary tract disease. *AJR Am J Roentgenol* 197: W551–W558.
 40. Kaneko K, Kimata T, Tsuji S, Ohashi A, Imai Y, Sudo H, Kitamura N, 2012. Measurement of urinary 8-oxo-7,8-dihydro-2-deoxyguanosine in a novel point-of-care testing device to assess oxidative stress in children. *Clin Chim Acta* 413: 1822–1826.
 41. Khuntikeo N et al.; CASCAP Investigators, 2015. Cohort profile: cholangiocarcinoma screening and care program (CASCAP). *BMC Cancer* 15: 459.