Urinary 8-Oxo-7,8-Dihydro-2′-Deoxyguanosine in Patients with Parasite Infection and Effect of Antiparasitic Drug in Relation to Cholangiocarcinogenesis

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Abstract

Parasite infection of Opisthorchis viverrini is a major risk factor for cholangiocarcinoma. Our previous immunohistochemical studies showed that O. viverrini infection induced oxidative DNA lesions in the bile duct epithelium during cholangiocarcinoma development. The current study assessed the levels of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative DNA lesion, in the urine and leukocytes of O. viverrini–infected subjects and cholangiocarcinoma patients. Forty-nine O. viverrini–infected patients, 55 cholangiocarcinoma patients, and 17 healthy controls were enrolled in the study. We measured 8-oxodG levels in the urine and leukocytes of these subjects using an electrochemical detector coupled to high-performance liquid chromatography. O. viverrini–infected patients were assessed before treatment and 2 months and 1 year after praziquantel treatment. Urinary 8-oxodG levels were significantly higher in cholangiocarcinoma patients (6.83 ± 1.00 µg/g creatinine) than in O. viverrini–infected patients (4.45 ± 0.25 µg/g creatinine; P < 0.05) and healthy subjects (3.03 ± 0.24 µg/g creatinine; P < 0.01) and higher in O. viverrini–infected subjects than in healthy subjects (P < 0.01). The urinary 8-oxodG levels in O. viverrini–infected patients significantly decreased 2 months after praziquantel treatment and were comparable with levels in healthy subjects 1 year after treatment. Urinary 8-oxodG levels were significantly correlated with leukocyte 8-oxodG levels, plasma nitrate/nitrite levels, and aspartate aminotransferase activity. In conclusion, this study, in addition to our previous studies, indicates that 8-oxodG formation by parasite infection may play an important role in cholangiocarcinoma development. Urinary 8-oxodG may be a useful biomarker to monitor not only infection but also carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2008;17(3):518–24)

Introduction

Chronic infection is well associated with some types of cancer through chronic inflammation (1-3). Large amounts of reactive oxygen species produced during inflammation are associated with an increased risk of human cancer (1, 2, 4). Oxidatively damaged DNA lesions have been suggested to contribute to several diseases, including cancers. 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an accepted marker for reactive oxygen species–induced DNA damage (5), is a mutagenic lesion leading to G → T transversions (6, 7) that are frequently found in tumor-associated genes in a variety of cancers (8, 9). Furthermore, some reports have shown elevated levels of urinary 8-oxodG in patients with different types of cancer (10-12). Therefore, urinary 8-oxodG may be a useful biomarker for cancer initiation and/or progression in cases where inflammation contributes to carcinogenesis. Measuring urinary 8-oxodG is noninvasive and therefore is an acceptable assay to biomonitor exposure to environmental carcinogens (13), including infection.

Parasite infection of Opisthorchis viverrini is classified as a group 1 carcinogen by the IARC (14) and is a risk factor for cholangiocarcinoma in Southeast Asia (15). O. viverrini infestation is endemic in Khon Kaen province, northeastern Thailand, and Khon Kaen has the highest incidence of cholangiocarcinoma in the world with an 80% to 90% occurrence rate (16). Several studies have shown that O. viverrini infection induces inflammation in both animal models (17, 18) and humans (19). Our previous studies (17, 20) on a hamster model showed that chronic inflammation induced by O. viverrini infection is a potential risk factor for cholangiocarcinoma, raising the question whether O. viverrini infection and cholangiocarcinoma development are reflected by the levels of 8-oxodG secreted in the urine.
To evaluate the usefulness of 8-oxodG as a factor to predict the risk of cholangiocarcinoma in *O. viverrini*–infected individuals, we measured 8-oxodG levels in the urine and leukocytes of healthy controls, *O. viverrini*–infected subjects, and cholangiocarcinoma patients using high-performance liquid chromatography coupled with an electrochemical detector. The effect of praziquantel, an effective drug for *O. viverrini*, on 8-oxodG levels was also assessed in the *O. viverrini*–infected group 2 months and 1 year after treatment. In addition, plasma nitrate/nitrite levels, metabolites of nitric oxide (NO), and aspartate aminotransferase (AST) activity, a biomarker of liver injury, were analyzed by spectrophotometry.

**Subjects and Methods**

**Subjects.** The protocol for this study was approved by the Ethics Group of the Human Research Committee (HE480316), Khon Kaen University, Thailand. The subjects in this study were divided into three groups: healthy subjects, *O. viverrini*–infected patients, and *O. viverrini*–infected cholangiocarcinoma patients. All participants volunteered for this study and gave informed consent. Both healthy control and *O. viverrini*–infected groups live in Khon Kaen province and have similar lifestyles, including smoking and alcohol drinking, although healthy control subjects have no habit of eating raw freshwater fishes, which transmit *O. viverrini*. Cholangiocarcinoma patients in hospital had no current alcohol drinking and smoking. Healthy subjects (8 males and 9 females) had an average age of 47.70 ± 8.01 years and were defined as persons who had never been infected with *O. viverrini*. Pretreated *O. viverrini*–infected patients (22 males and 27 females) had a positive *O. viverrini* egg count in stool, and the average age was 48.43 ± 13.11 years. Liver flukes were eliminated in the pretreated *O. viverrini*–infected group by praziquantel (a single dose of 40 mg/kg body weight).

Two months and 1 year after praziquantel treatment, patients whose stool was negative for *O. viverrini* eggs were defined as the posttreatment group. Obese, diabetic, and patients with chronic inflammation diseases, such as hepatitis virus and tuberculosis infections, were excluded from this study. Cholangiocarcinoma subjects (38 males and 17 females) were defined as patients who underwent surgical resection for cholangiocarcinoma at the Department of Surgery, Faculty of Medicine, Khon Kaen University, and this group had an average age of 54.02 ± 10.18 years. Cholangiocarcinoma was verified by histopathologic examination; other inflammatory diseases, such as cholangitis and hepatocarcinoma, were not included in this study. There was no significant difference in the average age of *O. viverrini*–infected subjects and healthy control subjects, but the average age of cholangiocarcinoma patients was higher than the other groups.

**Specimen Collection and Storage.** First morning urine specimens were collected, immediately frozen, and maintained at −80°C until analysis. After at least 12 h of fasting, peripheral blood was obtained by sterilized venipuncture, collected in tubes (10 mL/tube) with acid citrate dextrose, and centrifuged at 2,000 rpm at 4°C for 15 min, and the Buffy coat and plasma were isolated. The Buffy coat was treated with RBC lysis buffer [10 mmol/L Tris-HCl (pH 7.6), 5 mmol/L MgCl₂, and 10 mmol/L NaCl] to remove RBC and obtain a WBC pellet. WBC pellets were washed twice with sterile PBS. WBC pellets and plasma were stored at −80°C until use.

**Measurement of 8-oxodG in Urine.** 8-oxodG levels in the urine were determined by the method of Kasai et al. with slight modifications (21). Briefly, frozen samples were thawed and mixed completely. Urine samples were checked by Uropaper III (Eiken Kagaku K.K.) to exclude samples containing abnormal levels of WBC, RBC/hemoglobin, ketone, glucose, and protein. Thereafter, urine was centrifuged at 1,500 rpm for 5 min at 4°C, and the supernatant was transferred to a vial with 8-hydroxyguanosine (Cayman Chemical), a ribonucleoside marker. A 20 μL sample was injected into the high-performance liquid chromatography column (MCI GEL CA08F, 7 μm, 1.5 × 150 mm) at 58°C with a flow rate of 0.045 mL/min with the mobile phase consisting of 3 mmol/L H₂SO₄ and 2% (v/v) acetonitrile. The chromatogram was monitored by a UV detector (254 nm) depending on the relative elution position of the added marker to collect the 8-oxodG fraction. This fraction was injected into the second high-performance liquid chromatography (a Capcell Pak C18 column, 5 μm, 4.6 × 150 mm; Shiseido) at 30°C and a flow rate of 1 mL/min with the mobile phase consisting of 10 mmol/L NaH₂PO₄ and 5% (v/v) methanol. The 8-oxodG content was analyzed using an electrochemical detector (Coulochem II 5200A, ESA Biosciences) with the electrode voltage set to +300 mV. The molar ratio of 8-oxodG in each sample was measured based on the peak height of authentic 8-oxodG (Calbiochem) by the electrochemical detector. The urinary concentration of 8-oxodG was corrected by dividing the individual urinary creatinine concentrations. Urinary creatinine concentrations were determined using a creatinase method (Wako Pure Chemical Ind.).

**Measurement of 8-oxodG in Leukocyte DNA.** DNA was extracted from WBC pellets with a DNA Extractor WB Kit (Wako Pure Chemical Ind.). 8-oxodG levels in DNA were measured as described previously with slight modifications (22). DNA was dissolved in 20 mmol/L acetic acid buffer (pH 5.0) and digested to deoxyribonucleosides by incubating with 4 units nuclease P₁ (Wako Pure Chemical Ind.) at 37°C for 30 min followed by 0.6 units bacterial alkaline phosphatase (Sigma Chemical) at 37°C for 1 h in 0.1 mol/L Tris-HCl (pH 7.5). The deoxyribonucleosides were analyzed using electrochemical detector (Coulochem II 5200A, ESA Biosciences) coupled with high-performance liquid chromatography equipped with a Capcell Pak C18 column (4.6 × 150 mm; Shiseido). The mobile phase consisted of 100 mmol/L NaH₂PO₄, 5 mg/L EDTA, and 4% (v/v) methanol. The analysis was done at a column temperature of 15°C and a flow rate of 0.7 mL/min. The voltage of the electrode was set to +300 mV. The molar ratio of 8-oxodG to 2-deoxyguanosine in each sample was measured based on the electrochemical detector peak height of authentic 8-oxodG and the UV absorbance of 2′-deoxyguanosine at 254 nm.

**Analysis of Plasma Nitrate/Nitrite and AST.** The plasma levels of nitrate and nitrite were determined by the vanadium-based simple spectrophotometric method...
using the Griess reaction as described previously by Miranda et al. (23) with a minor modification. The assay was done in a standard flat-bottomed 96-well polystyrene microtiter plate. Nitrate concentrations in biological samples were measured after reducing to nitrite with the catalyst, VCl3. Plasma (100 μL) was deproteinized to reduce turbidity with 200 μL cold absolute methanol/diethyl ether (3:1, v/v) for 30 min at −80°C. The samples were centrifuged at 12,000 × g for 10 min, and the supernatant was analyzed for nitrate and nitrite. After 100 μL supernatant or standard nitrite was added to each well, 100 μL VCl3 was added followed by the immediate addition of 100 μL of the Griess reagents [premixed 50 μL of 2% sulfanilamide in 5% HCl and 50 μL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride]. The contents were vigorously mixed and the plate was incubated for 20 min at 37°C. The 540 nm absorbance was measured to assess the total amounts of nitrate and nitrite using sodium nitrite as the standard.

Plasma AST activities were determined spectrophotometrically using an automated analyzer.

**Statistical Analysis.** Data and transformed log-scale data were tested for data distribution. One-way ANOVA and Kruskal-Wallis tests were used to determine statistical significance in the normal and nonnormal distribution of data among cholangiocarcinoma patients, *O. viverrini*–infected patients, and healthy control groups. Significant differences between groups were analyzed by a Student’s *t* test or Mann-Whitney’s *U* test for normal distribution and nonnormal distribution data, respectively. The individual *O. viverrini*–infected patients were analyzed by the Friedman test and Wilcoxon signed-rank test to compare the degree of parasitic infection between the pretreatment and post-treatment groups at a 2-month and 1-year follow-up visit. Spearman’s rank correlation coefficient was used to analyze correlations for nonparametric data, whereas Pearson’s correlation coefficient was used for parametric data. *P* values less than 0.05 were considered statistically significant.

**Results**

Figure 1 shows urinary 8-oxodG levels in healthy, *O. viverrini*–infected, and cholangiocarcinoma subjects. The levels of urinary 8-oxodG in cholangiocarcinoma patients (6.83 ± 1.00 μg/g creatinine) were significantly higher than those in *O. viverrini*–infected patients (4.45 ± 0.25 μg/g creatinine; *P* < 0.05) and healthy subjects (3.03 ± 0.24 μg/g creatinine; *P* < 0.01; Fig. 1A). The urinary 8-oxodG levels in *O. viverrini*–infected patients were significantly higher than those in healthy subjects (*P* < 0.01). Figure 1B shows the effect of praziquantel treatment on 8-oxodG levels in *O. viverrini*–infected and healthy subjects. Two months after treatment, there was no significant difference between pretreated patients and healthy subjects. The urinary 8-oxodG levels in treated patients 1 year after therapy were significantly lower than those in pretreated patients (*P* < 0.01) and near levels in healthy control subjects. Figure 1C shows the time course of urinary 8-oxodG levels in *O. viverrini*–infected patients who were monitored 2 months and 1 year after *O. viverrini* removal treatment. 8-oxodG
levels in the urine gradually improved after treatment and were significantly decreased 2 months ($P < 0.05$) and 1 year ($P < 0.01$) after treatment.

Figure 2 shows leukocyte 8-oxodG levels in healthy, *O. viverrini*–infected, and cholangiocarcinoma subjects. Leukocyte 8-oxodG levels in cholangiocarcinoma patients (1.18 ± 0.22/10^5 dG) were significantly higher than those in healthy subjects (0.30 ± 0.02/10^5 dG; $P < 0.01$). Although a similar trend was observed with urinary 8-oxodG, the level of leukocyte 8-oxodG was not significantly different ($P = 0.052$) between *O. viverrini*–infected subjects (0.97 ± 0.10/10^5 dG) and cholangiocarcinoma patients (Fig. 2A). The levels of leukocyte 8-oxodG 2 months ($P < 0.01$) and 1 year ($P < 0.05$) after *O. viverrini* removal were significantly lower in the *O. viverrini*–infected group, but both levels were still significantly ($P < 0.01$) higher than levels in healthy control subjects. The time course of leukocyte 8-oxodG levels gradually decreased but was not significantly different by the Friedman test (Fig. 2C).

Figure 3 shows plasma nitrate/nitrite levels in healthy, *O. viverrini*–infected, and cholangiocarcinoma subjects. The plasma nitrate/nitrite levels in *O. viverrini*–infected and cholangiocarcinoma patients were significantly ($P < 0.01$) higher than those in healthy subjects (Fig. 3A). However, there was no significant difference between *O. viverrini* infection and cholangiocarcinoma. Two months after treatment, the plasma levels of nitrate/nitrite were significantly ($P < 0.01$) lower than those in pretreated subjects and reached levels similar to those in healthy subjects (Fig. 3B). Plasma nitrate/nitrite levels were significantly decreased 2 months ($P < 0.05$) and 1 year ($P < 0.01$) after *O. viverrini* eradication by praziquantel (Fig. 3C).

Figure 4 shows plasma AST activities in healthy, *O. viverrini*–infected, and cholangiocarcinoma subjects. AST levels in the *O. viverrini*–infected group were significantly ($P < 0.01$) higher than those in healthy subjects (Fig. 4A); however, these differences in AST levels may not be clinically relevant, suggesting that *O. viverrini* infection causes only slight liver damage. In contrast, plasma AST activity was significantly ($P < 0.01$) higher in cholangiocarcinoma patients than in healthy and *O. viverrini*–infected subjects, indicating that severe liver injury occurs with cholangiocarcinoma progression. Plasma AST levels significantly ($P < 0.05$) decreased 2 months after treatment (Fig. 4A), but the time course was not significantly different by the Friedman test (Fig. 4C). The average plasma AST level in *O. viverrini*–infected subjects was within the normal range.

Among healthy controls, *O. viverrini*–infected patients, and cholangiocarcinoma patients, there was a significant, positive correlation between urinary 8-oxodG and leukocyte 8-oxodG levels ($R = 0.382; P < 0.05$). The urinary 8-oxodG content significantly correlated with plasma levels of nitrate/nitrite ($R = 0.325; P < 0.05$) and AST ($R = 0.279; P < 0.05$). The leukocyte 8-oxodG content did not significantly correlate with plasma levels of nitrate/nitrite or AST.

**Discussion**

The present study was the first to show that 8-oxodG levels in urine and isolated leukocyte DNA were significantly decreased 2 months ($P < 0.05$) and 1 year ($P < 0.01$) after treatment.
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The plasma nitrate/nitrite levels were assessed in individual O. viverrini–infected patients treated with praziquantel. The plasma nitrate/nitrite levels were assessed in individual O. viverrini–infected subjects (n = 11), who could be followed for 1 year. #, P < 0.05; ##, P < 0.01, compared with pretreatment; $, P < 0.05; compared with 2 months after treatment. Mean ± SE.

Figure 3. Plasma nitrate/nitrite levels in cholangiocarcinoma patients, O. viverrini–infected patients, and healthy control subjects. A. Plasma nitrate/nitrite levels in healthy controls (n = 13), O. viverrini–infected patients (n = 43), and cholangiocarcinoma patients (n = 37). **, P < 0.01, compared with healthy subjects. B. Effect of praziquantel on plasma nitrate/nitrite levels in the O. viverrini–infected group [OV, pretreatment (n = 32); 2M, 2 months posttreatment (n = 31); 1Y, 1 year posttreatment (n = 34)]. **, P < 0.01, compared with healthy subjects; ##, P < 0.01, compared with pretreated O. viverrini–infected patients. C. Time course of plasma nitrate/nitrite levels in O. viverrini–infected patients treated with praziquantel. The plasma nitrate/nitrite levels were assessed in individual O. viverrini–infected subjects (n = 11), who could be followed for 1 year. #, P < 0.05; ##, P < 0.01, compared with pretreatment; $, P < 0.05; compared with 2 months after treatment. Mean ± SE.

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after treatment. Mean with pretreatment; $, P < 0.05; compared with healthy subjects; ##, P < 0.01, compared with healthy subjects; ##, P < 0.05; compared with healthy subjects. B. and cholangiocarcinoma patients (n = 31); 1Y, 1 year posttreatment (n = 34)]. **, P < 0.01, compared with healthy subjects; ##, P < 0.01, compared with healthy subjects; ##, P < 0.01, compared with healthy subjects; ##, P < 0.01, compared with healthy subjects.

Because these lesions have mutagenic properties, increased 8-oxodG levels may serve as a causative factor in the development of cholangiocarcinoma. In addition, immunohistochemical analyses on surgical specimens from cholangiocarcinoma patients (26) revealed that 8-oxodG in cancer tissue correlated with hypoxia-inducible factor-1α expression, which mediates inducible NO synthase expression, resulting in additional oxidative stress via mechanisms independent of infection. Interestingly, 8-oxodG levels in the urine were significantly higher in cholangiocarcinoma patients than in O. viverrini–infected subjects. The higher level of 8-oxodG in cholangiocarcinoma patients suggests that there is additional oxidative stress in cancer tissue and that urinary 8-oxodG may reflect a systemic burden both during O. viverrini infection and cholangiocarcinoma development. These results also indicate that urinary 8-oxodG may be a potential biomarker for predicting the risk of cholangiocarcinoma development.

Urinary 8-oxodG levels were significantly and positively correlated with plasma nitrate/nitrite levels. The accumulation of oxidative DNA damage may be associated with the production of reactive oxygen and nitrogen species in O. viverrini infection. Superoxide anion radical (O2·−) is derived from eosinophils (27) and macrophages (28) and dismutated to H2O2, which induces metal-dependent 8-oxodG formation (29). Our previous results showed that O. viverrini–infected hamsters had higher inducible NO synthase levels and increased 8-oxodG formation in the epithelial bile duct and in inflammatory cells at the site of infection (17, 30). In addition, inducible NO synthase expression in both inflammatory cells and bile duct epithelium was closely related with plasma level of nitrate/nitrite, the end products of NO (30). It is well known that NO reacts with O2·− to produce peroxynitrite (ONOO−), which also can induce 8-oxodG (31). Notably, a longitudinal observation of O. viverrini–infected individuals showed that plasma levels of nitrate/nitrite and 8-oxodG gradually decreased 2 months after parasite treatment and reached levels found in healthy control subjects after 1 year.

Our previous immunohistochemical analyses showed that 8-oxodG in the biliary epithelium of O. viverrini–infected hamsters resulted from the inflammatory response because 8-oxodG levels decreased after praziquantel treatment (32). A single praziquantel dose can eradicate O. viverrini as measured by egg counts in the stool; however, some inflammation with oxidative stress continued in the treated patients. Additional clinical approaches, such as anti-inflammation and antioxidative
strategies, may be required for several months after O. viverrini treatment.

It is known that the feeding and migrating activities of flukes contribute to hepatobiliary injury (33). In addition, immunopathologic processes may contribute to long-term hepatobiliary damage through 

\[ \text{O}_2^- \text{ and NO gene-} \text{rated from eosinophils and activated macrophages, resulting in 8-oxodG formation.} \]

In this study, urinary 8-oxodG levels positively correlated with AST activity in healthy subjects, O. viverrini–infected patients, and cholangiocarcinoma patients. The average AST level was significantly higher in O. viverrini–infected patients than healthy subjects but still within the normal range. AST activity in the plasma of cholangiocarcinoma patients was much higher than that in healthy subjects. This result is supported by a previous report showing that the number of 8-oxodG-positive hepatocytes was associated with AST activity in liver diseases and hepatocellular carcinoma (34). These findings can be explained by assuming that an increase in reactive oxygen species not only causes DNA damage but also induces hepatocyte injury, resulting in increased hepatobiliary enzyme activity.

A series of previous studies on both animal models and humans indicate that O. viverrini infection can induce the accumulation of potentially mutagenic modifications via reactive oxygen species production at inflammation sites and that these DNA lesions initiate carcinogenesis. We first showed that O. viverrini–induced oxidative stress could be detected by 8-oxodG that accumulates in leukocyte DNA and is excreted in the urine of O. viverrini–infected patients. Furthermore, 8-oxodG levels may increase during cholangiocarcinoma development in O. viverrini–associated cholangiocarcinoma patients, whereas eradication of O. viverrini decreases 8-oxodG levels. In the light of the presented data, we conclude that 8-oxodG levels, especially in the urine, may be a useful biomarker not only to monitor O. viverrini infection but also to assess the risk of O. viverrini–associated cholangiocarcinogenesis.

Acknowledgments

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References


Figure 4. Plasma AST activity in cholangiocarcinoma patients, O. viverrini–infected patients, and healthy control subjects. A. Plasma AST activity in healthy controls (n = 17), O. viverrini–infected patients (n = 47), and cholangiocarcinoma patients (n = 49). **, P < 0.01, compared with healthy subjects; #, P < 0.01, compared with O. viverrini–infected patients. B. Effect of praziquantel on the plasma AST activity in the O. viverrini–infected group [OV] pretreatment (n = 47); 2M, 2 months posttreatment (n = 51); 1Y, 1 year posttreatment (n = 28). **, P < 0.01, compared with healthy subjects; #, P < 0.05, compared with pretreated O. viverrini–infected patients. C. Time course of plasma AST activity in O. viverrini–infected patients treated with praziquantel. The plasma AST levels were assessed in individual O. viverrini–infected subjects (n = 11), who could be followed for 1 year. Mean ± SE.
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