Relationship Between Urinary 8-hydroxydeoxyguanine (8-OHdG) Levels and Clinicopathological Findings in Hepatobiliary Malignancies

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Abstract. *Background/Aim: Oxidative stress is defined as an imbalance between the pro-oxidant and antioxidant potential of cells leading to intracellular DNA damage. To clarify the oxidative stress response as a tumor marker, we investigated measurement of urinary 8-hydroxydeoxyguanosine (8-OHdG) levels in hepatobiliary diseases. Materials and Methods: Relationships between urinary 8-OHdG levels and clinicopathological factors were analyzed in 101 patients, including 84 with hepatobiliary malignancies, and 18 healthy volunteers. Co-existing biliary inflammation was detected in 8 patients. Results: Urinary 8-OHdG levels did not correlate with any clinical or liver functional parameters. The existence of inflammation and any tumor-related factor did not correlate with urinary 8-OHdG levels either. Urinary 8-OHdG levels were significantly higher in patients with benign and malignant diseases than in healthy volunteers ($p<0.05$), but not significantly different between benign and malignant diseases. Among patients with intrahepatic cholangiocarcinoma and gallbladder carcinoma, urinary 8-OHdG levels tended to be higher in patients with lymph node metastasis-positive than in those with lymph node-negative disease ($p=0.057$). Conclusion: The clinical significance of oxidative DNA damage and increases in its urinary metabolites in patients with hepatobiliary malignancies or inflammatory diseases remain unknown. Further studies are necessary to clarify the relationship between node metastasis and oxidative stress as a prognostic marker.*

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Complete (R0) resection is the only curable treatment for hepatobiliary malignancies and associated with patients' prognoses (1, 2). Even when curative surgery is completed, patients with increased preoperatively sensitive tumor markers show poor prognosis in these malignancies (3, 4). The normalization of these markers may be useful for defining curability (5). Most of these markers are analyzed in blood samples, the collection of which is associated with a risk of fine needle-stick accidents or contamination for examiners. Therefore, the examination of tumor markers using less invasive methods, such as urine or image diagnoses, is expected (6). However, non-invasive measurement of tumor markers in urine samples has not yet been extensively examined.

Oxidative stress is defined as an imbalance between the pro-oxidant and antioxidant potential of cells, which results either from the overproduction of reactive oxygen species (ROS), insufficient detoxification of ROS by antioxidants or a combination of both (7). ROS function as key metabolites that impair biological processes, resulting in various inflammatory diseases, including cardiovascular disease, diabetes mellitus, neurological disorders and cancers (8-10). In malignant diseases, severe oxidative stress that may influence the local reaction around the tumor is associated with more malignant behavior (11), while ROS are the primary cause of DNA damage (12). 8-hydroxydeoxyguanosine (8-OHdG), a hydroxyl product of deoxyguanosine that is produced by oxidative stress, is used as a biomarker of oxidative DNA damage (13). DNA base oxidation products, such as 8-OHdG, are excreted in urine (14). Oxidative DNA damage products have been shown to induce mutagenic guanine through the actions of ROS and lead to cell tumorigenesis (15). The deoxyguanine of 8-OHdG is

damaged by the active oxygen and extracellularly discharged in urine without being metabolized or degraded. Previous studies reported that immunohistochemical stain (16) or urinary concentrations of 8-OHdG, measured using competitive enzyme-linked immunosorbent assays, were higher in patients with liver or biliary cancers (17, 18). However, the relationships between the oxidative stress response in urine samples and clinicopathological findings in hepatobiliary malignancies have not yet been elucidated in detail. We hypothesized that the measurement of oxidative stress strongly reflects tumor development accompanied by local oxidative stress responses around the tumors in these malignancies.

In order to confirm our hypothesis, in the present study, we measured 8-OHdG levels in the urinary samples of 101 patients with hepatobiliary malignancies, as well as benign diseases, and 18 healthy volunteers. The relationships between 8-OHdG levels and clinicopathological factors were investigated in order to establish its significance as a potential tumor marker.

Materials and Methods

Patients. We examined 101 patients with hepatobiliary diseases who were admitted to the Department of Surgical Oncology, Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 2012 and May 2014. Urine samples were also provided by 18 healthy volunteers. The study design was approved by the Human Ethics Review Board of Nagasaki University Hospital (Reference number # 12012312). This study was supported by a Grant-in-Aid from the Research Promotion Bureau in Ministry of Education, Culture, Sports, Science and Technology, Japan (Reference number #23-521) between 2011 and March 2016. Informed consent for data collection was obtained from each patient during this period. Patients' data from electronic medical charts were analyzed and retrieved from the Department's database. Clinicopathological findings and tumor stages followed the Liver Cancer Study Group of Japan by the Classification of Primary Liver Cancer (19) and Classification of Biliary Tract Carcinoma by the Japanese Society of Biliary Surgery (20).

Measurement of urinary 8-OHdG. Prior to the morning meal, a 50-ml urinary sample from the first urination was collected from each patient. Samples were stored at –80˚C. 8-OHdG was assayed using an enzyme-linked immunoassay with New 8-OHdG Check ELISA (Japan Institute for the Control of Aging; NIKKEN SEIL Co., Ltd., Shizuoka, Japan). This kit detects 8-OHdG and a cross-reaction test was performed against 21 kinds of 8-OHdG analogs (guanosine(G),7-Methyl-G, 6-SH-G, 8-Bromo-G, dA, dC, dT, dI, dU, dG, O6-methyl-dG, 8-OHdA, guanine(Gua), O6-methyl-Gua, 8-OH-Gua, uric acid, urea, creatine, creatinine, 8-Sulfhydryl-G and 8-OH-G) according to the manufacturer's instructions. The detection ranges of 8-OHdG was between 0.5 and 200 ng/ml. Samples were reacted with a primary antibody at 37˚C for one hour and with a second antibody at 37˚C for one hour. A chromogenic reaction was performed at room temperature for 15 min.

Statistical analysis. Differences in categorical data between groups or prevalences were assessed using the Chi-square, Fischer's exact or Dunnett's multiple comparison tests. Relationships between two parameters were examined by calculating the Pearson's correlation coefficient. A two-tailed p-value of <0.05 was considered significant. Statistical Package for the Social Science (SPSS) version 18.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

The patients were 81 males and 38 females with a mean age of 68.7±10.5 years (range=23-84). The 18 healthy volunteers were all male and aged between 43 and 50 years. Seventeen patients had benign diseases. Malignant diseases included hepatocellular carcinoma in 47 patients, intrahepatic cholangiocarcinoma in 7, gallbladder carcinoma in 8 and extra-hepatic cholangiocarcinoma in 22. Benign diseases included benign liver tumors as hemangiomas in 3 patients, cholecystitis or cholangitis in 5 and others in 9. Co-existing biliary inflammation was detected in 8 patients.

Relationships with clinical parameters are shown in Table I. Urinary 8-OHdG levels did not correlate with any clinical or functional parameters or tumor markers. Table II shows the relationships between urinary 8-OHdG levels and clinicopathological findings or each tumor. Gender, background liver disease, viral status, the Child Pugh classification, existence of inflammation and any tumor-related factor did not correlate with urinary 8-OHdG levels. Urinary 8-OHdG levels were significantly higher in patients with benign and malignant diseases than in healthy volunteers ($p<0.05$) and were not significantly different between those with benign and malignant diseases ($p=0.218$).

Among patients with intrahepatic cholangiocarcinoma and gallbladder carcinoma, urinary 8-OHdG levels were slightly higher in patients with lymph node-positive disease than in those with lymph node-negative disease (3.71±2.41 *vs*. 12.99±5.14, respectively, in intrahepatic cholangiocarcinoma; $p=0.057$ and 4.49±1.30 *vs*. 10.62±3.98, respectively, in gallbladder carcinoma; $p=0.057$).

Discussion

To the best of our knowledge, this is the first study to investigate the relationships between the characteristics of hepatobiliary malignancies and urinary 8-OHdG levels. Increased expression of 8-OHdG reflects hydroxyl radical-mediated carcinogenesis, which influences the development of bladder cancer and is associated with a poor prognosis. (21). 8-OHdG levels in the DNA of leukocytes were previously reported to be significantly higher in bladder cancer patients than in healthy controls (22). Chiang *et al*. demonstrated that higher urinary 8-OHdG levels were associated with a greater risk of developing urothelial

Table I. *Correlations between urinary 8-OHdG levels and clinical data, laboratory data and tumor markers.*

	R value	*p*-Value (significance)
Age (years)	–0.131	0.172
Tumor size (cm)	0.194	0.075
ICGR15 (%)	–0.099	0.492
White blood cell count (/mm^3)	–0.148	0.134
Hemoglobin (g/dl)	–0.006	0.948
Total bilirubin (mg/dl)	–0.029	0.772
Albumin (g/dl)	0.127	0.201
AST (U/l)	–0.045	0.651
ALT (U/l)	–0.042	0.676
Prothrombin activity (%)	–0.021	0.839
Alkaline phosphatase (U/l)	–0.104	0.295
Cholinesterase (U/l)	0.005	0.964
Creatinine (mg/dl)	0.115	0.243
α-fetoprotein (ng/ml)	–0.084	0.580
PIVKA-II (mAU/ml)	0.090	0.538
CEA (ng/ml)	–0.151	0.212
CA19-9 (U/ml)	–0.068	0.644

ICGR15, Indocyanine green retention rate at 15 minutes; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PIVKA-II, protein induced by a vitamin K antagonist or agonist-II; CEA, carcinoembryonic antigen; CA, cancer antigen.

Table II. *Relationships between urinary 8-OHdG levels and clinicopathological data.*

	Urinary 8-OHdG	*p*-Value (significance)
Gender		
Male (n=81)	16.83±26.85	0.0061
Female (n=38)	7.94±7.92	
Background liver disease		
Normal liver (n=52)	18.43±32.24	ns
Chronic hepatitis (n=35)	12.77±12.51	
Cirrhosis (n=12)	10.29±8.62	
Obstructive jaundice (n=20)	10.57±7.11	
Diseases		
Healthy (n=18)	18.31±10.16	
Benign diseases	14.47±22.05*	
Liver tumor (n=3)	8.13±10.97	
Cholecysto (n=14) -choledocholithiasis	12.55±24.80	ns
Malignant	10.81±9.71**	
Hepatocellular carcinoma (n=47)	12.08±11.52	
Intrahepatic cholangiocarcinoma (n=7)	7.67±6.03	
Extrahepatic bile duct carcinoma (n=21)	9.88±7.36	
Gallbladder carcinoma (n=8)	8.50±4.30	
Viral status		
None (n=77)	15.54±25.84	ns
Hepatitis B (n=15)	15.09±13.07	
Hepatitis C (n=19)	9.91±10.04	
Child-Pugh classification		
A (n=101)	14.82±23.15	0.176
B (n=18)	9.37±12.42	
Existence of inflammation		
No (n=101)	14.18±21.95	0.060
Yes (n=18)	12.49±21.89	
Vascular invasion of tumors		
No (n=62)	14.12±26.65	0.832
Yes (n=23)	10.19±9.32	
Advanced tumor stage		
I, II (n=33)	10.20±9.62	0.446
III, IV (n=47)	15.22±30.23	
Node metastasis		
No (n=65)	11.09±10.66	0.592
Yes (n=13)	9.86±5.54	
Distant metastasis		
No (n=77)	10.88±9.96	0.888
Yes (n=2)	8.90±7.63	

ns, Not significant. *p=0.001 healthy *vs*. benign diseases, **p=0.029 healthy *vs*. malignant diseases.

carcinoma (23). However, the precise predictive value of 8-OHdG for the development of cancer remains unclear. Genetic damage in DNA may influence carcinogenesis by activating oncogenes or inactivating tumor suppressor genes. The increased accumulation of DNA damage products induces apoptosis (24). Impaired DNA repair results in the accumulation of various types of DNA lesions, including 8-OHdG. The lack of adequate 8-OHdG repair may be associated with any malignant disease (25).

In primary liver carcinoma, a relationship has been reported between chronic inflammatory hepatitis and carcinogenesis (26). Hepatitis accelerates DNA damage by inhibiting the recruitment of DNA repair complexes to damaged DNA sites, which results in hepatocarcinogenesis (27). Nishida *et al*. found elevated oxidative stress and marked epigenetic alterations during hepatocarcinogenesis (28). In an immunohistochemical analysis, the content of 8-OHdG in liver tissue may serve as a marker of oxidative stress and is currently used as a predictor of hepatocarcinogenesis (29). Jo *et al*. reported that oxidative stress enhanced the malignant potential of hepatocellular carcinoma (HCC) by stimulating angiogenesis through the activation of the vascular endothelial growth factor (VEGF) pathway (30). Higher 8-OHdG levels have been identified as a risk factor for the development of HCC in chronic hepatitis infections (31). In the present study, urinary 8-OHdG levels were significantly higher in patients with malignant diseases than in healthy volunteers. Healthy volunteers were selected among medical staff older than 40 years old because most patients in the present study were elderly and the potential contribution of occult DNA damage by aging was considered. Although smoking has been associated with higher 8-OHdG levels (32), only one healthy volunteer in the present study was a smoker and did not have higher urinary 8-OHdG levels. Furthermore, urinary 8-OHdG levels were significantly higher in patients than in healthy volunteers and, thus, the influence

of aging did not appear to be significant. However, in the present study, urinary 8-OHdG levels were not significantly different between patients with malignant and benign diseases and did not correlate with tumor-related parameters, laboratory data or inflammation. Therefore, oxidative DNA damage, reflected by urinary 8-OHdG levels, may be similar among inflammatory diseases, benign liver tumors and hepatobiliary malignancies. In this study, patients with severe inflammatory conditions, such as sepsis or multi-organ damage, were excluded. Urinary 8-OHdG levels were previously shown to be a sensitive marker for systemic inflammatory and lung diseases (33, 34). It has been reported that the oxidative stress response, as measured by diacron-reactive oxygen metabolite levels, was markedly increased in patients with infectious conditions who underwent lung transplantation (34). Enhanced oxidative stress responses are induced with exposure to infections. In our study, when the biliary inflammation was predominant, an increase in urinary 8-OHdG was not always observed. One patient underwent photodynamic therapy for bile duct carcinoma and developed transient cholangitis; however, urinary 8-OHdG levels only increased by 2 ng/ml. Therefore, biliary inflammation does not appear to increase urinary 8-OHdG levels more than pulmonary and other systemic infectious diseases.

By examining the relationship between tumor-related factors and urinary 8-OHdG levels in each carcinoma patient in our series, we found that these levels were increased in intrahepatic cholangiocarcinoma and gallbladder carcinoma patients with lymph node metastasis; however, not significantly. The relationship between node metastasis and urinary 8-OHdG levels is still unknown, although another oxidative stress marker, ROS, has been associated with lymph node metastasis in colorectal carcinoma (35). ROS may induce a wide type of cellular responses from proliferation to senescence or cell death, while a paradoxical biological response has been reported. Lymph node metastasis may be more related to prognosis than other tumor-related factors, particularly in patients with intrahepatic cholangiocarcinoma and gallbladder carcinoma (36, 37). In the metastatic process of advanced malignant tumors, the induction of oxidative DNA damage is possible in carcinoma or the surrounding normal tissues. In order to clarify the relationship between node metastasis and oxidative stress as a prognostic marker, further basic and clinical studies are needed.

In conclusion, we herein examined urinary 8-OHdG levels in 101 patients with hepatobiliary malignancies and benign diseases, as well as 18 healthy volunteers, and analyzed their relationships with clinicopathological factors. Urinary 8-OHdG levels were significantly higher in patients with malignancies than in healthy volunteers. The differences observed were not, however, statistically significant from those in patients with other benign hepatobiliary diseases and a correlation was not observed with any parameters. Urinary 8-OHdG levels were slightly higher in intrahepatic cholangiocarcinoma and gallbladder carcinoma patients with lymph node metastasis than in those without. The clinical significance of oxidative DNA damage and increases in its metabolites in hepatobiliary malignancies or inflammatory diseases remains currently unknown and, thus, warrants further scrutiny.

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