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8-Hydroxy-2'-deoxyguanosine expression predicts hepatocellular carcinoma outcome

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Abstract. Hepatocellular carcinoma (HCC) is characterized by increased oxidative stress and the production of 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is one of the main mutagenic modifications of DNA by oxidative stress. We analyzed the association of 8-OHdG with post-operative survival and revealed that low levels of 8-OHdG are associated with significantly shorter survival time. Moreover, levels of 8-OHdG were associated with HCC characteristics, including tumor size, tumor quantity, clinical staging, Child classification, portal vein thrombosis and ascites. These results suggest that oxidative damage is a useful prognostic marker in HCC when other clinical characteristics are present with 8-OHdG.

Introduction

Many reactive oxygen species (ROS), formed as by-products of energy production in aerobic animals, cause damage to macromolecules such as DNA, proteins and lipids, and are thus involved in a variety of biological processes, including aging, cancer and degenerative diseases (1). The hydroxyl radical is responsible for a number of base modifications, including thymine glycol and 8-hydroguanine (2). Oxidative damage to 2'-deoxyguanosine produces 8-hydroxy-2'-deoxyguanosine (8-OHdG), which induces G-C to T-A transversion in daughter DNA strands. In previous studies, 8-OHdG was shown to be a useful marker for assessing oxidative DNA damage (3,4), and levels of 8-OHdG in animal organ DNA have been used to assess the risk of carcinogenesis (5).

Hepatocellular carcinoma (HCC) is the fifth most frequent type of human cancer worldwide and the third highest cause of cancer-related mortality (6). More than 95% of HCC cases arise with a background of persistent inflammatory disease. The disease etiologies are diverse, although the hepatitis B virus (HBV) and hepatitis C virus (HCV) are implicated in the majority of cases (7).

Increased oxidative stress is associated with hepatitis viral infections and HCC progression (8,9). Free radical production is increased at the site of inflammation, resulting in lipid peroxidation and oxidative DNA damage, which are risk factors for HCC (10,11). 8-OHdG is well known as a high risk factor for HCC in chronic HCV infection (9,12). The prognosis for post-operative HCC patients remains poor due to high recurrence rates in spite of improved clinical detection methods for earlier diagnosis. Various factors, including tumor size, quantity of tumors, cell differentiation, venous invasion, advanced pTNM stage and degree of inflammation, have proven to be predictors for recurrence and prognosis for HCC patients. However, only a limited number of studies have focused on the relationship between oxidative markers and HCC prognosis (13-16). Since 8-OHdG has been used as a biomarker with prognostic significance for several types of cancer (17-19), we assessed the predictive power of 8-OHdG on HBV-HCC prognosis and examined the correlation between 8-OHdG and clinical characteristics of HBV-HCC. The post-operative HCC patients enrolled in this study were followed up with regular visits in our hospital. Tumor recurrence was diagnosed by laboratory tests combined with contrast-enhanced computed tomography (CT) or contrast-enhanced magnetic resonance imaging (MRI), and the overall survival rate was recorded.

Materials and methods

Tissue collection. We obtained histologically confirmed hepatic cancerous tissues from 110 HCC patients, including 95 patients with HBV associated with HCC (HBV-HCC) and 15 patients with HCV associated with HCC (HCV-HCC), who received an HCC resection operation between January 2002 and June 2004 at the Hepatobiliary Surgery Department in the Fourth Hospital of Hebei Medical University, China, according to the guidelines of the human tissue research committee at the hospital. Tissues were fixed in formalin (10%) immediately after resection, dehydrated in absolute ethanol and embedded in

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paraffin. Serial sections (5 μm) were prepared for immunohistochemical analysis. All patients received and signed consent forms and all procedures were supervised and approved by the hospital's Human Tissue Research Committee.

Measurement of 8-OHdG in hepatocarcinoma cancer tissues. The 8-OHdG levels were measured by hepatic immunohistochemical staining. Briefly, tissue sections were incubated for 2 days at 4˚C with anti-8-OHdG antibody (dilution 1:100; Abcam, Cambridge, UK) and then reacted with biotinylated secondary anti-mouse IgG antibody for 1 h at room temperature. Streptavidin was added and the color was developed with 3,3'-diaminobenzidine (DAB).

The level of 8-OHdG was measured as described (13). Two pathologists counted the number of 8-OhdG-stained hepatocytes in 10 random fields (magnification, x400). We calculated the percentage of positively stained cells, which was termed the 8-OHdG label index (LI), in each field and graded them as follows: low, LI <50%; high, LI >50% (Fig. 1).

Statistical analysis. The survival data were analyzed by the Kaplan-Meier method, differences were determined by the log-rank test and the prognostic significance of clinical characteristics was assessed by the multivariate Cox proportional hazard model. Differences in clinical characteristics between the high and low LI groups were compared with the χ^2 test. P≤0.05 was considered to be statistically significant and all calculations were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA).

Results

Clinical characteristics of HBV-HCC patients. A total of 110 patients were enrolled in this study and a review was conducted every 3 months for 3 years. Seven patients were lost during follow-up; one HBV-HCC patient in the first year, one HCV-HCC and three HBV-HCC patients in the second year and one HCV-HCC and one HBV-HCC patient in the third year. The remaining 103 patients (90 HBV-HCC and 13 HCV-HCC) were assessed. Adjuvant chemotherapy or radiation therapy were not administered following HCC resection. The data collected during the 3-year follow-up were analyzed for clinical characteristics as described in Materials and methods. No difference was observed for gender, age, tumor size, tumor quantity, Child classification, clinical stage, portal vein thrombosis or ascites between HBV-HCC and HCV-HCC patients, so we combined them for further analysis. Age was not a significant predictor for post-operative survival time, but gender, tumor size, tumor quantity, tumor stage, Child classification, portal vein thrombosis and ascites were positively correlated with survival time (Table I). The 3-year survival rate was 14.8% for the 18 female patients and 36.7% for the 85 male patients. Patients with tumor diameter ≥5 cm had a significantly shorter survival time than those with tumor diameter <5 cm. As expected, patients at different stages of the disease had significantly different 3-year survival rates as determined by the log-rank test: stage I-II, 44.1% and stage III-IV, 3.9%. Patients with portal vein thrombosis had a significantly shorter survival time than patients without venous invasion. Child classification and ascites were associated with HCC survival.

Table I. Univariate analysis of clinical characteristics associated with post-operational survival in HCC patients.

Characteristics	No. of cases	5-yr survival rate (%)	P-value
Gender			0.023
Male	85	36.7	
Female	18	14.8	
Age (years)			0.964
≤55	43	35.4	
>55	60	31.7	
Quantity of tumors			0.001
Single	68	39.2	
Multiple	35	19.4	
Diameter of tumor (cm)			0.023
<5	33	47	
≥5	70	25.3	
TNM classification			0.000
I-II	73	44.1	
III-IV	30	3.9	
Child classification			0.000
A	85	37.7	
B+C[a]	18	7.2	
Portal vein thrombosis			0.000
Yes	46	9.7	
No	57	51.1	
Ascites			0.040
Yes	44	28	
No	59	33.4	

[a]As there were only 4 Child C patients, B and C patients were combined to form one group.

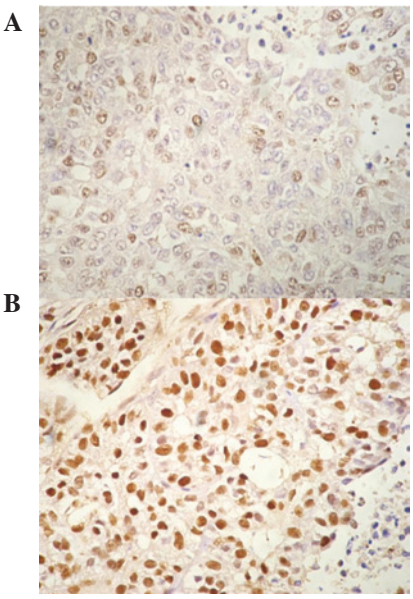

Figure 1. 8-OHdG immunostaining in HCC tissues with (A) low expression and (B) high expression. Cells with a brown-stained nucleus are regarded as positive. Original magnification, x400.

Table II. Multivariate analysis of prognostic factors associated with post-operational survival in HCC patients with the Cox proportional hazard model.

Factors	Relative risk	95% CI	P-value
Gender	1.153	0.589-2.258	0.678
Tumor quantity	2.057	1.152-3.674	0.015
Size of the tumor	0.779	0.427-1.423	0.417
TNM classification	0.369	0.158-0.862	0.021
Child classification	1.735	1.026-2.932	0.040
Portal vein thrombosis	2.934	1.376-6.256	0.005
Ascites	1.420	0.835-2.415	0.195
8-OHdG	0.152	0.051-0.455	0.001

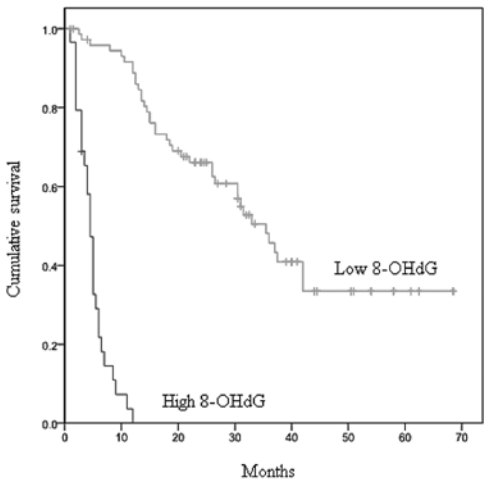

Figure 2. Significant survival difference between HCC patients in low and high 8-OHdG groups (p=0.000).

Table III. Association of 8-OHdG with HCC clinical characteristics.

Characteristics	8-OHdG		P-value
	Low	High	
Gender			0.091
Male	64	21	
Female	10	8	
Age (years)			0.692
≤55	30	13	
>55	44	16	
Numbers of the tumor			0.001
Single	56	12	
Multiple	18	17	
Diameter of the tumor			0.044
<5	46	24	
≥5	28	5	
TNM classification			0.000
I-II	67	6	
III-IV	7	23	
Child classification			0.000
A	70	15	
B+C	4	14	
Portal vein thrombosis			0.000
Yes	19	27	
No	55	2	
Ascites			0.003
Yes	25	19	
No	49	10	

The data demonstrated that gender, tumor size, tumor quantity, tumor stage, Child classification, venous invasion and ascites are good predictors of HCC outcome.

8-OHdG level predicts prognosis of HCC patients. The immunoreactivity of 8-OHdG observed in HBV-HCC tissues occurred mostly in the nuclei (Fig. 1). We graded the LI (the percentage of HCC cells containing 8-OHdG) as low or high for the 3-year analysis and the cumulative survival rate was plotted as a Kaplan-Meier curve (Fig. 1). As shown in Fig. 2, the log-rank test revealed a markedly significant difference (p=0.000) between the low and high LI groups demonstrating the usefulness of the oxidative DNA damage marker 8-OHdG in predicting the prognosis of HBV-HCC.

Multivariate analysis of prognosis factors for HBV-HCC. We used a multivariate analysis, including the predictors mentioned above, with the Cox proportional hazard method to evaluate the effect of this unusual oxidative DNA marker on the predictive power of traditional clinical parameters. The analysis revealed that the 8-OHdG level, tumor quantity, clinical stage and portal vein thrombosis were independent risk factors for the post-operative survival rate of HBV-HCC patients (Table II). Among the predictors, 8-OHdG had the strongest link to the HBV-HCC survival rate, with a hazard ratio of 0.152 (95% CI, 0.051-0.455). Univariate and multivariate analyses suggested that the 8-OHdG level in HCC tissue DNA is one of the best predictors of the post-operative survival rate of HCC patients.

Correlation between 8-OHdG and other predictors of HBV-HCC. The correlation of clinical characteristics and 8-OHdG levels was evaluated in HCC patients using the χ^2 test and the results are shown in Table III. Gender and age were not correlated with 8-OHdG levels, whereas tumor size, tumor quantity, clinical stage, Child classification, portal vein thrombosis and ascites were significantly correlated with 8-OHdG expression. The data suggest that 8-OHdG is capable of modifying tumor development in combination with other predictors.

Discussion

We used immunohistochemical methods with a monoclonal antibody against 8-OHdG with paraffin-embedded HCC sections for the assessment of oxidatively damaged DNA in

HBV-HCC patients. The 8-OHdG generated as a result of oxidative DNA damage is capable of inducing mutations in genes and is closely involved in hepatocarcinogenesis (5,9,12,20). We demonstrated that 8-OHdG expression levels in cancer tissues were associated significantly with the 3-year post-operative survival rate in HBV-HCC patients. The 8-OHdG levels were a risk factor for hepatocarcinogenesis and a good marker for predicting the outcome of post-operative HBV-HCC patients. Earlier studies suggested that oxidative DNA damage was associated with cancer progression, including cell proliferation, apoptosis, genetic instability and chemoresistant phenotypes (21-24), whereas the functional significance of persistent oxidative stress in HCC tissue remains unknown.

Consistent with the results of earlier studies, we found that tumor quantity, venous invasion, advanced pTNM staging and Child classification were independent predictors for HCC. We found these predictors to be correlated with 8-OHdG expression levels, suggesting that there is an intrinsic interaction between oxidative stress and the characteristics associated with HCC outcome. Miyake *et al* revealed an association between 8-OHdG expression and tumor size, clinical stage and venous invasion in the progression of renal cell carcinoma (17). Whether oxidative damage is the cause of these clinical characteristics, thus affecting tumor progression indirectly, or whether it modifies the tumor progression directly in cooperation with these characteristics requires further analysis.

The degree of inflammation is associated with hepatocarcinogenesis and outcome in HCC patients (13,25). Hepatic oxidative stress is associated with hepatic inflammation in viral hepatitis patients (9,26). It is unclear whether oxidative stress is the cause or the consequence of liver injury, but it has been demonstrated that the oxidative stress directly activates Kuffer cells, causing the release of inflammatory and profibrogenic cytokines, including tumor necrosis factor-α and transforming growth factor-β (27). Oxidative stress has been shown to have a significant association with hepatic fibrosis (28). Further clinical trials are required to evaluate the effect of anti-oxidative therapy on the survival of post-operative HBV-HCC patients.

In conclusion, we report that 8-OHdG is a novel prognostic factor in HCC. The strong association between 8-OHdG expression and poor patient survival together with the correlation between clinical staging, Child classification, tumor size and venous invasion, suggests a significant role for oxidative stress in HCC carcinogenesis and tumor behavior. Further studies are required to clarify whether extensive oxidative stress in highly malignant tumors is a prerequisite for carcinoma progression or secondary changes during carcinogenesis.

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