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ORIGINAL RESEARCH ARTICLE

The expression and significance of 8-hydroxydeoxyguanosine in breast cancer patients' blood, urine and cancer tissue

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ABSTRACT

Objective: To explore the expression and clinic significance of 8-OHdG in breast cancer. **Methods**: Pre-operative serum 8-OHdG levels were detected with an enzyme-linked immunosorbent assay in a well-defined series of 173 breast cancer patients. 8-OHdG expression in cancer cells from 150 of these patients was examined by immunohistochemistry. The HPLC-ECD method is used to determine 8-OHdG concentration in urine. **Results**: The serum 8-OHdG levels and immunohistochemical 8-OHdG expression were in concordance with each other ($P < 0.05$, $r = 0.163$). Breast cancer patients with negative 8-OHdG immunostaining show lower survival rate according to the multivariate analysis ($P < 0.01$). This observation was even more remarkable in ductal carcinomas (n = 140) patients ($P < 0.001$). A low serum 8-OHdG level was associated statistically significantly with lymphatic vessel invasion and a positive lymph node status. Comparison of 8-OHdG concentration in urine of breast cancer patients and healthy women was statistical significance ($P < 0.01$). **Conclusion**: Low serum 8-OHdG levels and a low immunohistochemical 8-OHdG expression were associated with an aggressive breast cancer phenotype. In addition, negative 8-OHdG immunostaining was an independent prognostic factor for breast cancer-specific death in breast carcinoma patients. Using 8-OHdG concentration in urine to predict DNA damage resulting from breast cancer can provide good biological indicators for detecting harm in early breast cancer.

Keywords: 8-OHdG; Enzyme-linked Immunosorbent Assay; Immunohistochemistry; Reactive Oxygen Species

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1. Introduction

ROS (reactive oxygen species) is a metabolite of normal cells, which can act on pyrimidine, purine and chromatin proteins, resulting in gene base modification and gene mutation. These reactions interact with oncogenes and may lead to cancer formation[1].

Because the life of ROS is very short, for example, the life of the most harmful -OH is estimated to be less than 1 ns, it is difficult to detect ROS directly. Therefore, the most effective method to detect ROS is to use antibodies to neutralize the "footprint" of oxidative damage[2]. 8-hydroxydeoxyguanosine (8-OHdG) is a specific marker of 2-deoxyguanosine damage caused by ROS attacking DNA. The molecular weight of 8-OHdG is 283.2. It is formed after the hydroxyl radical (-OH) in oxygen-containing radical (ROS) attacks the DNA base guanine and is removed from the DNA chain after enzyme repair. It is water-soluble and can be excreted from the body through urine[3]. 8-OHdG is one of the biomarkers of oxidative stress. It can be detected by immunohistochemistry, enzyme-linked immunosorbent assay and HPLC-ECD[4]. In this study, we analyzed the level of 8-OHdG in

serum and urine, 8-OHdG expression in tissue, and combined with clinicopathological parameters to evaluate the feasibility of 8-OHdG as a predictor and prognostic factor of breast cancer.

2. Materials and methods

2.1 Data collection

A total of 173 breast cancer patients admitted to the First Affiliated Hospital of Gannan Medical University from 1982 to 2011 were selected. All cases were histologically confirmed as breast cancer, and the staging criteria were based on the tumor staging established by WHO. None of them received any treatment before blood collection. All the patients were female, including 140 cases of ductal carcinoma, 25 cases of lobular carcinoma and 8 cases of other types of breast cancer. The average follow-up time of this study was 40.5 months. Serum samples were stored in polystyrene tubes at –80 °C. Pathological wax blocks of 150 patients were randomly selected from the 173 patients for immunohistochemical examination. Urine of 60 normal women of the same age group who had not suffered from gynecological diseases or received treatment in the past were collected as the control group, and the concentration of 8-OHdG in urine was analyzed. This study was approved by the medical ethics committee of the First Affiliated Hospital of Gannan Medical University.

2.2 Using ELISA to detect the expression level of 8-OHdG in blood of breast cancer patients

The level of 8-OHdG in serum was determined by ELISA (ELISA kit was purchased from Shanghai Esha Biotechnology Co., Ltd., as well as all the following reagents). Anti 8-OHdG monoclonal antibody was used. Blood samples were pretreated with a microporous filter. 200 µL serum was added to each test tube, centrifuged at 140 rpm for 30 min; the supernatant was taken, added with primary antibody. Another test tube was added with samples, plate vibrated, and incubated at 4 °C for the night. Each tube was rinsed with 250 µL rinse solution for 3 times, followed by secondary antibody, plate vibration, and incubation at room temperature for 1 h. Then, each test tube was added with 100 µL reaction stopper, and plate vibrated, and the absorbents were measured at 450 nm on the panel display. The standard curve was used to calculate the amount of 8-OHdG in the samples.

2.3 Using immunohistochemistry to detect 8-OHdG expression in breast cancer tissue samples

Paraffin blocks were conventionally sliced, de-waxed with xylene, dehydrated with alcohol of different gradients, and heated with 10 mm citric acid in a microwave oven for 10 min. The slices were cooled at room temperature, soaked in 3% hydrogen peroxide in methanol for 15 min, and incubated overnight at 4 °C. The incubation solution was 1:125 primary antibody (8-OHdG), colorant was 1:400 biological secondary antibody and avidin-biotin-peroxidase complex. Aminobtetraethyl lead was used as color-changing material, while xylitol was used for the staining count.

The intensity of 8-OHdG in cells was divided into four groups: – negative (nuclear staining <5%), + weak (nuclear staining 5%–20%), ++ medium (nuclear staining 21%–80%), +++ strong (nuclear staining >80%). For statistical analysis, staining results were divided into negative (–) and positive (+, ++, +++).

2.4 Examination of the concentration of 8-OHDG in urine by HPLC-ECD

Urine samples from breast cancer patients were collected by catheterization before surgery, and those from normal women were collected by natural urination. Urine samples were stored in a refrigerator at –80 °C. Adjust pH value of urine to 4.5 by HCl solution. 5 mL urine was put into a 15 mL centrifuge tube, centrifuged at 1,500 rpm for 5 min, and 2 mL supernatant was taken and added to the first extraction tube. Add 10 mL MeOH into the extraction tube; add 5 mL distilled water; add 10 mL buffer A; add 3 mL buffer A for washing, and add 3 mL 5% MeOH into buffer A for washing, and collect it in a 15 mL centrifuge tube. Add the collected solution to the second extraction tube. Add 1.5 mL 20% MeOH into buffer A for washing, and collect it in a 15 mL centrifuge tube. Remove MeOH in a vacuum concentrator and condense it for 1.5 h. Use buffer A for quantification to 1 mL.

Inject 100 µL of it into HPLC-ECD for analysis.

2.5 Statistical analysis

Software spss 15.0 was used for statistical analysis. Spearman's test, Mann-Whitney U-test and Pearson x^2 were used to calculate the results of ELISA, immunohistochemistry and HPLC-ECD, respectively. The survival rate was analyzed by the survival curve. External factor analysis was performed by lox regression analysis, $P < 0.05$ means the difference is statistically significant.

3. Results

3.1 Expression of 8-OHdG in breast cancer tissue samples

8-OHdG immunohistochemical staining was located in the nucleus (**Figure 1**). Among all patients, 147 patients had positive 8-OHdG immunohistochemistry; among the patients with intraductal carcinoma, there were 120 patients with positive 8-OHdG immunohistochemical expression. The distribution of immunohistochemical staining is shown in **Table 1**. According to Spearman's test, the expression of 8-OHdG in serum was positively correlated with that in tissue ($P < 0.05$, $r = 0.163$).

Table 1. 8-OHdG immunohistochemical results

Group	n	8-OHdG expression							
		–		+		++		+++	
		n	%	n	%	n	%	n	%
All the patients	173	26	15.0	26	15.0	73	42.1	48	27.7
Ductal carcinoma patients	140	20	14.2	27	19.2	59	42.1	34	24.2

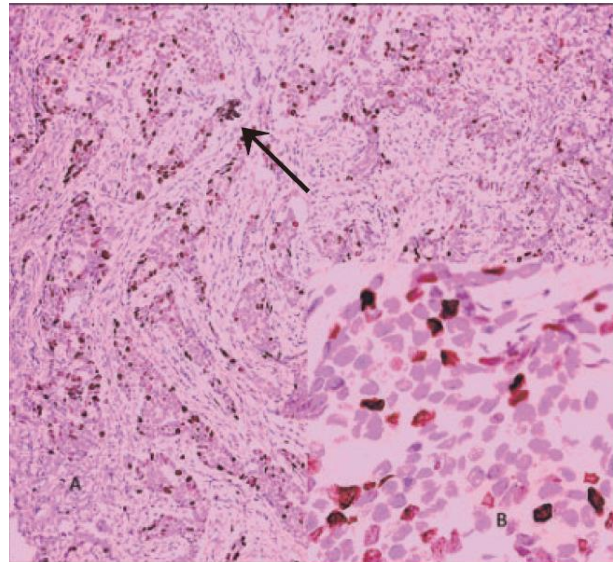


Figure 1. Strong positive 8-OHdG expression in breast cancer. Note: A: ×10; B: ×40; arrows indicate positive cells.

3.2 Relationship between the expression of 8-OHdG in blood and the biological characteristics of breast cancer patients

Among patients with breast cancer, the level of serum 8-OHdG in patients with lymphatic metastasis and lymph node metastasis is relatively low. When comparing the biological characteristics of tumor in all patients, there was statistical significance between the two characteristics of lymphatic metastasis and the number of lymph node metastasis and the level of serum 8-OHdG ($P < 0.05$) (**Table 2**).

Table 2. Statistical relationship between 8-OHdG and biological characteristics of breast cancer

Clinical information	**8-OHdG value**	***P* value**	**Clinical information**	**8-OHdG value**	***P* value**	**Clinical information**	**8-OHdG value**	***P* value**
T staging			Lymphatic metastasis			Progesterone receptor		
1	0.18 ±0.13	0.09	Positive	0.12 ±0.09	< 0.05	Positive	0.16 ±0.12	0.35
2 ~ 4	0.15 ±0.11		Negative	0.17 ±0.13		Negative	0.19 ±0.13	
N staging			Vascular metastasis			Ki-67		
0	0.18 ±0.12	< 0.05	Positive	0.10 ±0.04	0.18	0 ~ 2	0.16 ±0.12	0.41
1 ~ 2	0.15 ±0.13		Negative	0.17 ±0.13		3	0.19 ±0.14	
Grading			Estrogen receptor			Her-2		
1 ~ 2	0.17 ±0.12	0.37	Positive	0.16 ±0.12	0.24	Positive	0.15 ±0.09	0.14
3	0.17 ±0.13		Negative	0.19 ±0.14		Negative	0.17 ±0.13	

3.3 Relationship between 8-OHdG and clinicopathological parameters in breast cancer tissue

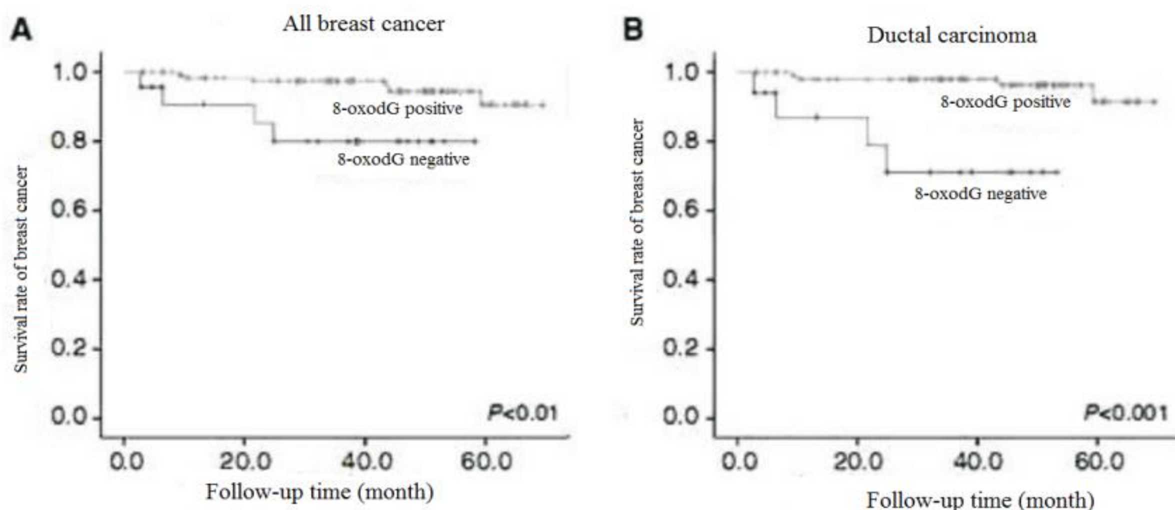
Compared with patients with positive 8-OHdG immunohistochemical expression, those with negative expression had a higher risk of death. Survival

rate data are shown in **Table 3**. In survival factor analysis, negative 8-OHdG is an independent prognostic factor in patients with low survival rate. The results of survival curve analysis showed that there was a significant difference in disease-free survival time between positive and negative 8-OHdG positive (see **Figure 2**).

Table 3. Relationship between 8-OHdG and biological characteristics of breast cancer

Clinical information	n	Average survival time/month	*P* value	Clinical information	n	Average survival time/month	*P* value
T staging				Progesterone receptor			
1	110	68.7	<0.01	Positive	116	67.5	<0.05
2 ~ 4	63	63.5		Negative	57	64.3	
N staging				Ki-67			
0	97	70.4	<0.01	0 ~ 2	127	69.6	<0.001
1 ~ 2	76	62.6		3	46	56.7	
Tissue grading				Her-2			
1 ~ 2	110	70.0	<0.01	Positive	22	60.8	0.21
3	63	62.1		Negative	151	67.6	
Lymphatic metastasis				Histological type			
Positive	16	55.6	0.16	Ductal	140	66.4	0.33
Negative	151	67.9		Others	33	67.7	
Vascular metastasis				8-OHdG expression			
Positive	9	58.2	0.09	Positive	127	66.9	<0.01
Negative	158	67.6		Negative	23	49.5	
Estrogen receptor				8-OHdG expression in ductal carcinoma			
Positive	140	69.0	<0.01	Positive	106	67.4	<0.001
Negative	33	55.9		Negative	17	42.1	

Note: only 167 patients with lymphatic and vascular metastasis were collected, and the other 6 patients in the early stage had no relevant information.



A: all breast cancer ($P < 0.01$); B: ductal carcinoma ($P < 0.001$)

Figure 2. Survival curve analysis.

3.4 Concentration of 8-OHdG in urine of breast cancer patients and healthy women

The 8-OHdG concentration in urine and the correction by creatinine and body weight of breast cancer patients and healthy women were compared (**Table 4**). The concentration of 8-OHdG in the two

groups was statistically significant ($P < 0.01$). The mean 8-OHdG concentration in urine of breast cancer patients was (81.81 ±74.4) nmol, which was higher than that of healthy women (33.14 ± 18.1) nmol ($P < 0.01$). Corrected by creatinine in urine, the mean 8-OHdG concentration in urine of breast cancer patients was (16.39 ± 17.3) $\mu mol \cdot mol^{-1}$, still higher than that of healthy women (4.70 ± 7.1) $\mu mol \cdot mol^{-1}$ ($P < 0.01$). After corrected by body weight, the mean value of 8-OHdG/kg in urine of breast cancer patients was (676.82 ± 608.0) $pmol \cdot kg^{-1}$, which was also higher than that of healthy women (286.37 ± 160.7) $pmol \cdot kg^{-1}$ ($P < 0.01$).

Table 4. Comparison of 8-OHdG concentration in urine between breast cancer patients and the control group/$\bar{x} \pm s$

Group	8-OHdG /nmol	8-OHdG/Creatinine /$\mu mol \cdot mol^{-1}$	8-OHdG /$pmol \cdot kg^{-1}$
Breast cancer n = 173	81.81 ±74.4 (15.45–354.27)	16.39 ±17.3 (1.64–90.01)	676.82 ± 608.0 (131.09–3163.11)
Control group n = 60	33.14 ± 18.1 (16.86–101.81)	4.70 ±7.1 (0.94–53.22)	286.37 ± 160.7 (127.62–808.53)
***P* value**	<0.01	<0.01	<0.01

4. Discussion

ROS is a by-product of normal cell metabolism and may also be produced by stimulation of foreign substances. 8-OHdG is the product formed after ROS attacks DNA. The formation of 8-OHdG is easy to cause errors in DNA replication, resulting in gene mutation, and then cancer; at the same time, it will be removed from the DNA strand after repaired by repair enzyme *in vivo*[5].

Studies have pointed out that when cells are attacked by carcinogens, they may produce some oxygen-containing free radicals, which may cause oxidative damage to nucleic acids; when these products are attacked by oxygen-containing radicals, they cause more than dozens of products of nucleic acids oxidative damage, of which 8-OHdG is the most representative; and because 8-OHdG will cause the error of deoxyribose insertion during DNA replication, G → T conversion occurs[6]. It is found that 8-OHdG can be used as a biological index related to mutation formation or cancer formation[7]. Through this study, it is found that 8-OHdG negative staining in breast cancer tissue may be an independent prognostic factor for breast cancer patients with poor prognosis. The low level of 8-OHdG expression in serum and tissue may be a strong feature of breast cancer invasion. This study found that there was a positive correlation between oxidative stress and serum 8-OHdG level in breast cancer cells.

In this study, the low expression of 8-OHdG in breast cancer tissue and the low level of 8-OHdG in preoperative serum were significantly correlated with the prognosis of breast cancer. This correlation is more obvious in ductal carcinoma. Ductal carcinoma is an important histological subtype of breast cancer with different prognosis. Therefore, more accurate prognostic factors need to be identified. According to our experimental results, negative 8-OHdG expression and low serum 8-OHdG levels in tumor tissue are independent prognostic factors of low survival rate in breast cancer patients.

The low level of serum 8-OHdG is a sign of weak DNA repair after oxidative damage, or the improvement of antioxidant defense function relative to ROS. The main repair enzyme of 8-OHdG is DNA glycosylase 1, whose function is very important to prevent base pair G → T mutation[8]. ROS can damage DNA glycosylase 1 and cannot cleave damaged guanine, resulting in the decrease of 8-OHdG level in extracellular fluid[9]. The improvement of antioxidant defense in tumor tissue provides advantages for cancer cell growth by avoiding apoptosis and ROS induced necrosis. Excessive antioxidant enzymes will prevent the interaction between ROS and DNA, thus reducing the formation of 8-OHdG in tissue[10]. Translation factor Nrf 2 is an up-regulator of multifunctional antioxidant enzymes, which can remove ROS from cells. On the other hand, Nrf 2 upregulation is very common in drug-resistant cancer cells. It can provide cancer cell growth advantage in the tumor treatment stage[11]. Although 8-OHdG is relatively less studied in breast cancer patients, Nrf 2 up-regulated, and antioxidant enzyme inducers and resistance may explain the poor prognosis of patients with low8-OHdG in the initial stage of 8-OHdG.

By comparing the concentration of 8-OHdG in

the urine of breast cancer patients and healthy women, the former was 81.81 nmol, which was significantly higher than that of the latter, 33.14 nmol, with statistical difference ($P < 0.001$). The same results were obtained after adjustment by creatinine and body weight. There has been no studies on the correlation between 8-OHdG in the urine of breast cancer patients and healthy women. From the results of this study, it has been found that there was a statistically significant correlation between the concentration of 8-OHdG in urine and breast cancer patients. Therefore, the concentration of 8-OHdG in urine can be used to predict the DNA damage caused by breast cancer and provide good biological indicators for the early damage of breast cancer.

We believe that the expression of immunohistochemical 8-OHdG expression in breast cancer patients is related to the level of 8-OHdG in plasma. The decrease of 8-OHdG in plasma and breast cancer cells indicates the increase of invasiveness, especially in ductal cancer. Negative immunohistochemical 8-OHdG expression is an independent predictor in breast cancer patients. These results provide a standard for judging the prognosis of breast cancer, and provide an important preliminary study for further better treatment of tumors.

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgements

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