

8-OHdG and Nrf2 Protein are Expressed Consistently in Various T Stages of Invasive Breast Carcinoma

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Abstract

Objective: Oxidative stress prompts breast cancer cells to adapt by raising the lethal threshold and enhancing the antioxidant mechanism, thereby enabling survival and continuous proliferation that facilitates tumor progression. *Nrf2* and *8-OHdG* are indicative of oxidative stress activity and impact the progression of breast cancer. We aimed to analyze the expression of *Nrf2* and *8-OHdG* in various T stages of breast cancer in our hospital. **Methods:** This observational study employed a cross-sectional design and included patients with invasive breast carcinoma of no special type diagnosis from histopathology examination who underwent modified radical mastectomy without neoadjuvant chemotherapy at Dr. Soetomo General Academic Hospital between January 2019 and December 2022. Medical records and paraffin blocks that met these criteria were obtained. *8-OHdG* and *Nrf2* were assessed using immunohistochemistry. **Result:** There was no significant difference and correlation between *8-OHdG* ($p=0.578$) and *Nrf2* ($p=0.694$) expression with various T stages of IBC-NST and no significant correlation between *8-OHdG/Nrf2* expression and T stage ($p=0.242$ and 0.625 respectively). **Conclusion:** Consistent expression of *8-OHdG* and *Nrf2* in various T stages of breast cancer represents a continuation of the oxidative stress process in breast cancer that is not influenced by the tumor size. The existence of consistent oxidative stress at all tumor sizes (T stage) stimulates breast cancer cells to continue proliferating.

Keywords: Breast cancer- invasive breast carcinoma of no special type- *8-OHdG*- *Nrf2*

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Introduction

Breast cancer is a prevalent malignancy in women, with 66,271 new cases recorded in Indonesia in 2022 [1]. The increased proliferation of cancer cells results in the consequent accumulation of elevated reactive oxygen species (ROS) and oxidative stress, which are harmful to the cells. Increased oxidative stress and hypoxic conditions prompt breast cancer cells to adapt by raising the threshold for lethal levels of oxidative stress, enhancing antioxidant mechanisms and angiogenesis. These adaptations enable breast cancer cells to withstand elevated oxidative stress and continue to proliferate [2-5].

Both normal and cancer cells undergo cellular metabolism, which induces an imbalance between pro-oxidant and antioxidant, a phenomenon of oxidative stress [6]. Oxidative stress affects carcinogenesis, including the promotion, progression, and aggressiveness of breast cancer, through increased cell proliferation. Oxidative stress accumulates at a significant level in the late stage of breast cancer [7, 8]. A practical method to detect oxidative stress is the detection of 8-hydroxy-2-deoxyguanosine (*8-OHdG*), the product of DNA damage by ROS [9]. Low expression of *8-OHdG*, a prognostic factor for

poor prognosis, is a robust indicator of breast cancer invasion and is more prevalent in ductal carcinoma type [10-13]. This phenomenon can be attributed, in part, to a higher antioxidant activity in breast cancer tissue [14]. The primary regulator of the antioxidant enzyme, nuclear factor erythroid 2-related factor 2 (*Nrf2*), is highly expressed in breast cancer. Upregulation of *Nrf2* and its associated enzymes reduce *8-OHdG* expression and counteract the deleterious effects of oxidative stress, leading to the progression and metastatic potential of breast cancer [11, 14].

A few studies analyzed the different amounts of oxidative stress in various tumor sizes, whether higher or lesser in bigger tumor sizes. We aimed to analyze the expression of *Nrf2* and *8-OHdG* in various T stages of breast cancer.

Materials and Methods

Sample collection

Samples from 49 patients who had not received neoadjuvant chemotherapy, underwent modified radical mastectomy (MRM) and were identified as having invasive breast carcinoma of no special type (IBC-NST)

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by histopathology examination at Dr. Soetomo General Academic Hospital between January 2019 – December 2022 were used in this study. Using the breast cancer T stage categorization by the World Health Organization (WHO), samples were divided into four groups: T1 (14 cases), T2 (14 cases), T3 (14 cases), and T4 (7 cases). Before the study, all sample re-examination was conducted.

Immunohistochemistry

Formalin-fixed paraffin-embedded blocks (FFPE) were cut 4–6 µm thick. Deparaffinized using xylol and rehydrated using absolute, 96%, 80%, and 70% alcohol for 5 minutes were performed. Slides were washed in running and distilled water for 5 minutes, followed by 15 minutes of placing at room temperature in 3% H₂O₂ in methanol and rewash for 5 minutes with distilled water. Slides were then warmed for 30 minutes at 95 °C in a deep decloaking chamber using a Diva Decloaker with PH 7 for *8-OHdG* and *Nrf2*. PBS was used for 5-minute washing. The background snipper was dripped for 15 minutes, followed by the primary antibodies. The primary antibodies were mouse monoclonal antibody against *8-OHdG* (e-8) sc-393871 (Santa Cruz Biotechnology) at 1:100 dilution and rabbit monoclonal antibody IgG against *Nrf2* bsm-52179R (Bioss Antibodies) at 1:100 dilution. After 60 minutes of incubation at room temperature, slides were washed with PBS for 5 minutes. The universal link was dripped for 20 minutes, washed with PBS, dripped with the HRP Trekavidin label for 15 minutes, and washed again with PBS. Then, diaminobenzidine (DAB) was dripped. Slides were incubated at room temperature for five minutes, washed with running water, and then Mayer's Hematoxylin was added. Slides were then washed with running water and soaked in water for 5 minutes. Dehydration in 80%, 96%, and absolute alcohol was performed then. The slides were then placed in xylol and continued by covering each slide with cover glass. Two pathologists, unaware or blinded to the T stage of the samples, independently assessed the immunostaining results using the Olympus CX31 binocular microscope. Assessment and settlement of disagreements between observers were performed using a double-headed microscope.

Scoring

The nuclei of tumor cells were examined for *8-OHdG* and *Nrf2* immunostaining localization. There were four staining intensity levels: negative, weak, moderate, and strong. Histochemical scoring assessment (H-score), which was the result of combining the values of all intensity levels that were overlaid on the tumor cells (negative, weak, moderate, and strong) multiplied by the percentage of overlaid tumor cells (1-100%). The H-score formula is stated below:

$$\text{H-score} = (0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)$$

The results were then summed to obtain a range of H-score values from 0 to 300. The H-score ranges were grouped into 4: negative = 0 (0-49), weak = 1 (50-99),

moderate = 2 (100-199), and strong = 3 (200-300) [15-18].

Statistical analysis

IBM SPSS Statistics 26.0.0.0 was used for statistical analysis. Using the Kruskal-Wallis and Spearman's correlation tests, the difference and correlation of *8-OHdG* and *Nrf2* expression in various T stages of breast cancer were assessed. Results were significant if the p-value is < 0.05.

Results

Patient characteristics

Patients with IBC-NST in this study had an average age of 52.69 years. The oldest patient was 84 years old, and the youngest was 28 years old. Patients' ages were divided into seven groups with 10-year time intervals. The highest prevalence of 18 patients (36.73%) was in the age group of 41-50 years old. The T2 stage in the population of this study had 89 cases, followed by the T4 stage with 42 cases, the T3 stage with 36 cases, and the T1 stage with 29 cases. T1 stage in this study was found most in the 41-50 years old age group with 6 cases (12.24%), T2 stage in the 51-60 years old age group with 7 cases (14.29%), T3 stage in the 41-50 years old age group with 5 cases (10.20%), and T4 stage in the 41-50 years old age group with 4 cases (8.16%) (Table 1).

Using the Nottingham modification Bloom and Richardson grading system, the histology grading of breast cancer in this study was divided into three grades: grade 1 – grade 3. The most common grade in this study was grade 3, with 28 cases (57.14%), followed by grade 2, with 19 cases (38.78%), and grade 1, with 2 cases (4.08%) (Table 1).

8-OHdG expression

8-OHdG expression was observed in all T1-T4 groups in this study. The expression of *8-OHdG* was evaluated and examined using H-score (Figure 1). *8-OHdG* expression varied across all T stage groups. The most prevalent score was 1 in 22 out of 49 samples. The T1, T2, and T4 stage group's highest score was 1 (6, 8, and 4 cases, respectively), and the T3 stage group's highest score was 2, with 5 cases (35.71%) (Table 2). There was no significant difference between *8-OHdG* expression at various T stages of breast cancer (p-value = 0.578) (Table 2), and no significant correlation was observed between *8-OHdG* expression and T stage (p-value = 0.242) in this study (Table 4).

Nrf2 expression

The H-score was used to evaluate *Nrf2* expression of *Nrf2* (Figure 2). The most prevalent score was 1 in 23 out of 49 samples. The T2 and T3 groups' highest scores were 1 (9 and 7 cases, respectively), the T1 stage group's highest score was 0 with 5 cases (35.71%), and the T4 stage group's highest score was 1 with 3 cases (42.86%) (Table 3). There was no significant difference in *Nrf2* expression between various T stages of breast cancer (p-value = 0.694) (Table 3), and no significant correlation was observed between *Nrf2* expression and T

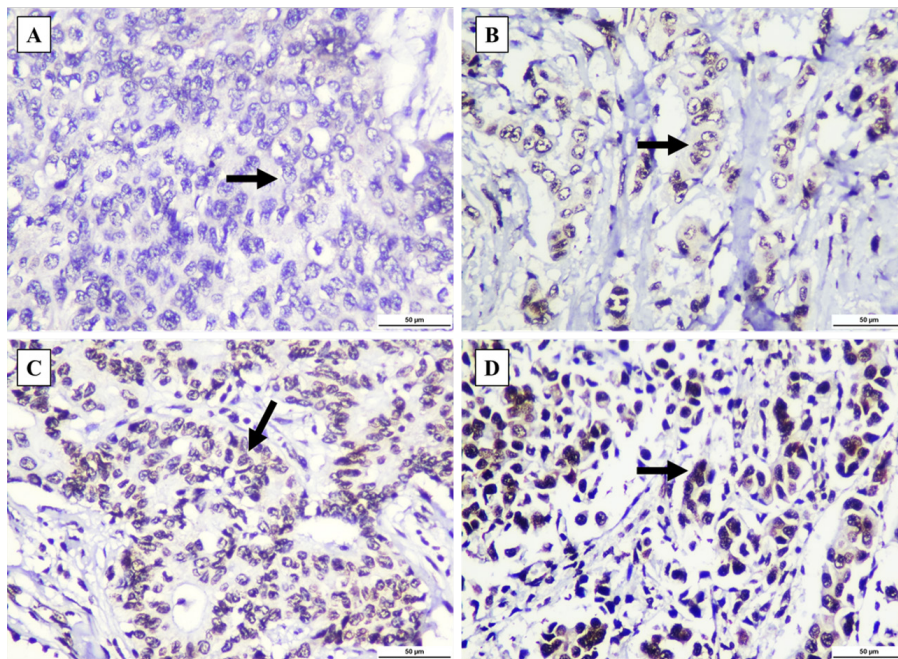


Figure 1. *8-OHdG* Staining was Observed in Invasive Breast Carcinoma of No Special Type (IBC-NST) Tumor Cell's Nuclei (black arrow). (A) Negative staining. (B) Weakly positive staining. (C) Moderately positive staining. (D) Strongly positive staining. All figures were captured at 400x magnification. Scale bar: 50 µm.

stage (p-value = 0.625) in this study (Table 4).

Discussion

This study found that the T2 stage was the most common. Research conducted by Yahya et al. at Universitas Airlangga Hospital in 2022 found that the most common tumor size in their research was between 2-5 cm or stated as T2 stage [19]. Age is a significant risk factor, and the global incidence of breast cancer increases in conjunction with advancing age and subsequently decreases after menstruation [20, 21]. The incidence of

breast cancer is higher in Asian women aged under 50 years old than in Western women, with the peak incidence occurring at 40-50 years old in Asia and 60-70 years old in Western countries [22]. The increasing incidence of breast cancer may be caused, among other things, by increased exposure to risk factors among younger individuals, as well as variations in quality of care and public awareness about breast cancer in Western countries [23]. The elevated risk of breast cancer observed in premenopausal women and the gradual decline in postmenopausal age may be attributed to estrogen and progesterone exposure [20].

The grade of breast cancer is classified based on Nottingham modification Bloom and Richardson grading system, which evaluates tumor cell aggressiveness by tubular formation, nuclear pleomorphism, and mitosis count; the histology grading of breast cancer in this study was divided into 3 grades [24]. The most common grade in this study was grade 3. A study by Anwar et al. in Sardjito Hospital in 2019 found that 78.5% of 1.259 cases of breast cancer in young women were grade 3, and a study by Syarti et al. in 2020 found that 44.8% of all breast cancer cases with spiculated mass in mammography examination were grade 3 [25, 26]. Grade has a high prognostic value, with grade 3 having 31% of the 10-year relapse risk, grade 1 being 7%, and grade 2 being 14% [27]. SEER data indicate that the 5-year survival rate in women with breast cancer limited to the breast and has size <1 cm is 99%, 89% in 1-3 cm, and 86% in 3-5 cm. Large tumor size significantly predicts regional lymph node involvement and skin or chest wall invasion correlates with poor prognosis [20].

8-OHdG in this study was expressed in all breast cancer cells, regardless of the T stage. Liu and Liu, in 2021, stated that *8-OHdG* expression in breast cancer cells was not significantly correlated with T stage but was instead correlated with lymph node involvement (N

Table 1. Clinicopathology Characteristics

Characteristics	T1	T2	T3	T4	Total n (%)
Population	29	89	36	42	196
Sample	14	14	14	7	49
Age					
21-30	0	0	0	1	1 (2.04)
31-40	3	1	2	0	6 (12.24)
41-50	6	3	5	4	18 (36.73)
51-60	1	7	3	1	12 (24.49)
61-70	3	2	2	1	8 (16.33)
71-80	1	1	1	0	3 (6.12)
81-90	0	0	1	0	1 (2.04)
Tumor location					
Right breast	5	8	9	2	24 (48.98)
Left breast	9	6	5	5	25 (51.02)
Grade					
1	1	0	1	0	2 (4.08)
2	9	6	1	3	19 (38.78)
3	4	8	12	4	28 (57.14)

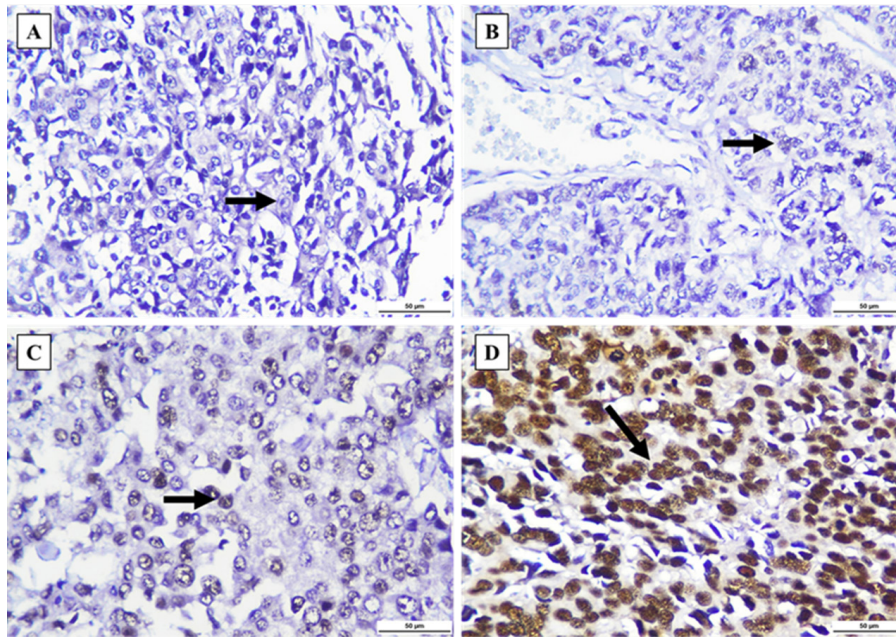


Figure 2. *Nrf2* Staining was Observed in Invasive Breast Carcinoma of No Special Type (IBC-NST) Tumor Cell's Nuclei (black arrow). (A) Negative staining. (B) Weakly positive staining. (C) Moderately positive staining. (D) Strongly positive staining. All figures were captured at 400x magnification. Scale bar: 50 µm.

Table 2. H-score for *8-OHdG* Expression

<i>8-OHdG</i>		T stage								Total (n)	p-value
		T1		T2		T3		T4			
H-score	Interpretation	n	%	n	%	n	%	n	%		
0	Negative	2	14.29	1	7.14	4	28.57	2	28.57	9	0.578
1	Weak	6	42.86	8	57.14	4	28.57	4	57.14	22	
2	Moderate	5	35.71	5	35.71	5	35.71	1	14.29	16	
3	Strong	1	7.14	0	0	1	7.14	0	0	2	
Total (n)		14	100	14	100	14	100	7	100	49	

*Kruskal-Wallis test, p <0.05 was considered statistically significant

Table 3. H-score of *Nrf2* Expression

<i>Nrf2</i>		T stage								Total (n)	p-value
		T1		T2		T3		T4			
H-score	Interpretation	n	%	n	%	n	%	n	%		
0	Negative	5	35.71	3	21.43	4	28.57	1	14.29	13	0.694
1	Weak	4	28.57	9	64.29	7	50	3	42.86	23	
2	Moderate	4	28.57	1	7.14	3	21.43	2	28.57	10	
3	Strong	1	7.14	1	7.14	0	0	1	14.29	3	
Total (n)		14	100	14	100	14	100	7	100	49	

*Kruskal-Wallis test, p <0.05 was considered statistically significant

stage) [13]. On the contrary, Pande et al. [28] stated that there was a correlation between *8-OHdG* and tumor size. Negative *8-OHdG* staining in breast cancer tissue is an

independent prognostic factor of poor prognosis and low survival rate [11, 12, 14]. A positive correlation exists between *8-OHdG* levels and oxidative stress in breast

Table 4. *8-OHdG* and *Nrf2* Expression Correlation Analysis

Parameter	p-value	Correlation Coefficient
Correlation of <i>8-OHdG</i> expression with T stage	0.242	-0.170
Correlation of <i>Nrf2</i> expression with T stage	0.625	0.072
Correlation of <i>8-OHdG</i> and <i>Nrf2</i> with T stage	0.027	-0.317

*Spearman's correlation test, p <0.05 was considered statistically significant

cancer cells [13]. 8-OHdG is a biomarker of oxidative stress and oxidative DNA damage [29, 30]. The primary 8-OHdG repair enzyme is DNA glycosylase 1 (OGG1), which prevents G to T base pair mutation and further DNA damage [31]. ROS can inactivate OGG1, resulting in the inability to cleave the damaged guanine, reducing the amount of 8-OHdG [32]. Antioxidant enzyme activity can inhibit the interaction between ROS and DNA, thereby inhibiting the formation of 8-OHdG [33]. Compared with non-invasive breast lesions, 8-OHdG expression is significantly diminished in breast cancer [11].

Nrf2 in this study was also expressed in all breast cancer cells, regardless of the T stage. Lee et al. [34] found that *Nrf2* expression was not correlated with the T stage. However, nuclear immunoreactivity of *Nrf2* was correlated with histological grade and *Ki67* index. Onodera et al. [35] found that *Nrf2* expression was marginally correlated with T stage. The discrepancies in *Nrf2* correlation may be caused by the activation of *Nrf2* through alternative pathways, apart from the Keap1-*Nrf2* pathway [34].

Nrf2 plays a significant role in maintaining cellular homeostasis and activating cytoprotective genes [36-38]. Kelch-like ECH-associated protein 1 (Keap1) interacts with *Nrf2* and controls the stability of *Nrf2* [39]. The Keap1-*Nrf2* pathway is a primary defense mechanism against oxidative stress and a significant hallmark of the regulation and development of breast cancer [38, 40]. The complexity and nature of cancer cell heterogeneity, along with the contribution of *Nrf2*, are currently being widely researched. Available data indicating the significant role of *Nrf2* in the progression of breast cancer are still limited [41]. The activation of *Nrf2* that activates HIF-1 α increases the progression and angiogenesis in breast cancer. HIF-1 α , the primary transcription factor for adaptive hypoxia condition, regulates several genes, including those involved in metabolic processes, angiogenesis (FGF and VEGF), and apoptosis (Bcl2, Bax, and p53). Reducing *Nrf2* levels will decrease HIF-1 α mRNA, reducing breast cancer cell proliferation [38]. Although not statistically significant, the expression of *Nrf2* was lower in normal breast tissue than in breast cancer tissue. *Nrf2* expression in the cytoplasm was identified in almost all cell lines, whereas *Nrf2* expression in the nucleus was significantly higher in breast cancer cells [42]. The data demonstrated that all breast cancer types exhibited elevated *Nrf2* levels in both the nucleus and cytoplasm. This finding shows that, regardless of the immunophenotype of breast cancer cells, tumor cells require augmented antioxidant protection [43]. The function of *Nrf2* activity in breast cancer cells is reflected by nuclear *Nrf2* immunoreactivity. In contrast, the importance of the *Nrf2* activation pathway in breast cancer tissue is indicated by the wide distribution of *Nrf2* immunoreactivity [35].

In breast cancer, a reduction in 8-OHdG levels was observed as tumor aggressiveness and progressivity increased. Enhanced antioxidant defenses facilitate cancer cell growth by inhibiting ROS-induced necrosis and apoptosis. Increased antioxidant enzyme activity impedes the interaction between ROS and DNA, thereby reducing 8-OHdG formation. A reduction in 8-OHdG levels in

breast cancer cells indicates enhanced invasive properties, particularly in ductal carcinoma [13]. *Nrf2* activation is increased in the tumor microenvironment, facilitating the accumulation of *Nrf2* in the nucleus. This accumulation triggers tumor cell protection by transcribing protective genes that protect tumor cells against oxidative stress. In breast cancer, this often leads to tumor progression, resistance to anticancer treatment, especially under hypoxic conditions, and poor prognosis [42, 44]. Nuclear accumulation of *Nrf2* will increase the transcription of genes involved in antioxidants, such as genes related to antioxidant response elements (ARE), namely glutathione reductase (GR), catalase (CA), NAD(P)H dehydrogenase quinone 1 (NQO1), superoxide dismutase (SOD), heme oxygenase-1 (HO-1), and glutathione peroxidase (GPX), as well as pro-proliferation, such as *IGF1*, *NPNT*, *Notch1*, *VEGFC*, *SHMT*, *PSPH*, *PSAT1*, and *PHGDH*, which will increase breast cancer cell proliferation. *Nrf2* also degrades 8-OHdG through the upregulation of OGG1 [45].

Consistent with the findings and theory described above, our study showed that in breast cancer, 8-OHdG and *Nrf2* were expressed in all T stages. This represents the continuation of the oxidative process in all stages of breast cancer, and oxidative stress is required for cancer cell growth. This has opened a new possibility that antioxidant interventions could potentially have beneficial effects on breast cancer patients by interfering with the oxidative stress process.

In conclusion, based on the results and data analysis, 8-OHdG and *Nrf2* are consistently expressed in various T stages in breast cancer, indicating that the continuation of the oxidative stress process is not influenced by the size of the tumor (T stage). The existence of consistent oxidative stress at all tumor sizes (T stage) stimulates breast cancer cells to continue their proliferation.

Author Contribution Statement

All authors contributed to the conception and design of the study. Fira Soraya was responsible for data collection, analysis, interpretation, and manuscript writing. Willy Sandhika and Priangga Adi Wiratama developed the concept and critically revised the manuscript for important intellectual content, reviewed the text and manuscript writing, and approved the final version. All authors read and approved the final manuscript..

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Ethical Declaration

Approval from the Research Ethical Committee of Dr. Soetomo General Academic Hospital, dated November 4th, 2023, with number 0824/KEPK/XI/2023, was obtained.

Conflict of Interest

All of the authors declare no conflict of interest.

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