Expression of γ-H2AX and patient prognosis in breast cancer cohort

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Abstract
H2AX phosphorylation is a novel marker of DNA double-stranded breaks. In the present study, we assessed the γ-H2AX expression, its association with other clinicopathologic characteristics, and the prognosis in a cohort of 97 patients with breast cancer. Ninety-seven specimens of tumor tissue and 77 adjacent normal tissues from patients with breast cancer were examined. All patients underwent modified radical mastectomy or local tumor resection without lymph node dissection. γ-H2AX expression was assessed by standard immunohistochemistry. Patients were followed after surgery for a mean duration of 70.1 ± 18.7 months (range, 6-93 months). The γ-H2AX staining was positive in 27 (27.8%) patients. The positive rates of H2AX were 26.0% and 2.6% in tumor tissue and adjacent normal tissues, respectively. γ-H2AX positive status was negatively associated with TNM staging, with 24 positive cases (32.4%) in TNM staging I-II, while no positive cases in TNM staging III-IV (P = 0.026). Sixteen patients (16.5%) died during the follow-up. No significant association between γ-H2AX expression and patient survival was detected. The unadjusted HR (hazard ratio) for γ-H2AX positive was 0.84 (95% CI: 0.27, 2.60). In TNM staging subgroup analysis, death only occurred in γ-H2AX negative patients. Our study is the first study to demonstrate that expression of γ-H2AX is associated with TNM staging. Due to the small sample and limited follow-up time, we did not observe a significant association between γ-H2AX and patient survival. γ-H2AX expression could be a potential biomarker for cancer diagnosis and prediction, and further studies are in need.

KEYWORDS
breast cancer, DNA damage response, H2AX, prognosis

1 | INTRODUCTION

The DNA damage repair pathway is activated upon damage to DNA caused by various causes, including ionizing radiation, hypoxia, reactive oxygen species as well as certain chemicals.¹ In these cases, double-stranded breaks (DSBs) can occur, and both DNA strands can be cleaved and be lethal for the cells.² The DSBs can initiate genomic instability and frequently predispose to cancer development.³ One of the first events in reaction...
to activation of the DSBs repair pathway is phosphorylation of histone H2AX. Histone H2AX becomes rapidly phosphorylated at serine 139 residues from the N terminus, referred to as γ-H2AX, in the DSB sites and at the break spots in the chromosomes. Phosphorylation of H2AX facilitates the assembly of DNA repair proteins, including p53 at the sites containing DNA double-strand breaks and damaged chromatin. Thus, γ-H2AX is widely used as a surrogate marker of DSBs. Unrepaired DSBs, indicated by retention of irradiation induced γ-H2AX foci, have predictive value in tumors as a biomarker for sensitivity to radiotherapy. The transcription factor p53, which also plays important roles in the cellular responses to DNA damage, regulation of cell cycle and genomic stability, is mutant in many cancers. It was suggested that H2AX and p53 play synergistic roles in DNA damage responses and tumor suppression. The activation of DNA damage response may affect the survival of malignant cells. Endogenous expression of γ-H2AX has also been observed in cancer tissues and even in their precursor lesions, suggesting a role for activated DNA damage repair in tumorigenesis.

Breast cancer is the most common cancer in women and about one in eight (12%) will develop invasive breast cancer in their lifetime in the United States. It is estimated that 246,600 new breast cancer cases would be diagnosed and 40,450 would die in 2016. Recently, the association between γ-H2AX expression and BRCA1 mutation and prognosis has been found in breast cancers. However, the endogenous expression of γ-H2AX in breast cancer and adjacent normal tissue, patient’s characteristics and the prognosis of γ-H2AX expression have, to the best of our knowledge, remained inconclusive. In this study, we assessed the endogenous DNA DSBs, revealed by the γ-H2AX expression assay in tumor cells as well as in adjacent normal tissues in patients with breast cancer, and investigated the correlation of γ-H2AX with other pathoclinical parameters and the overall survival (OS) of patients.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 97 inpatients with breast cancer in the Department of Oncology, Shanghai Hospital between 2010 January and 2013 July were studied. Among the enrolled cases, 85 patients underwent modified radical mastectomy, 12 cases underwent local tumor resection without lymph node dissection. All patients were female. None had radiotherapy, chemotherapy or molecular targeted therapy before surgery. Among patients undergoing modified radical mastectomy, 29 cases had lymph node metastasis and 56 cases had no lymph node metastasis. This research was approved by the ethics committee of the hospital, and informed consent was signed by the patients or their authorizers.

2.2 | Tissues

Ninety-seven postoperative tumor specimens and 77 normal adjacent tissues (>20 mm away from cancerous margin) were collected. The diameters of 59 tumor tissues were ≤20 mm, and the diameters of 38 cases were >20 mm. All tissues were preserved in paraffin wax. The pathological diagnosis was confirmed by at least two pathologists. All histological grades were issued according to WHO (2003) Breast tumor histological classification. Histological grades were reported according to SBR (Scarff-Bloom-Richardson) grading standards. TNM staging was made according to the 2009 AJCC seventh edition of breast cancer staging criteria.12

2.3 | Tissue microarrays

Representative areas from each tumor and adjacent normal tissues were chosen in hematoxylin and eosin-stained sections; these areas were subsequently punched out from paraffin blocks, to construct tissue microarrays (TMAs) using a Manual Tissue Arrayer from Beecher International Inc (model MTA-1; Sun Prairie, Wisconsin). One to five 2 mm-wide tissue cores from each tissue were chosen. All cases were distributed over eight TMA recipient paraffin blocks, which were incubated at 56°C for 5 minutes to stick recipient and donor paraffin together.

2.4 | Immunohistochemistry

Immunostaining of TMAs was performed on 4 μm-thick sections, deparaffinized in dimethylbenzene, and hydrated in a series of graded alcohol dilutions. Slides were boiled in a microwave at 650 W for 20 minutes, immersed in a high-pH target-retrieval solution (K8004; Dako, Glostrup, Denmark) and subsequently cooled at room temperature for 20 minutes. Endogenous peroxidase was blocked with 3% H2O2 in methanol. Sections were incubated with primary antibodies against phospho-H2AX (Ser139; rabbit monoclonal; 20E3; 1:100 dilution; Cell Signaling Technology, Danver, MA). Slides were then incubated with Envision/HRP (Agilent, Madison, WI) for 30 minutes, following which the antigen-antibody complex was visualized using DAB (Agilent) as chromogen for 10 minutes. All the sections were lightly counterstained with hematoxylin before mounting. All the series included both positive controls (ie, tissues known to express the relevant antigen) and negative controls (ie, duplicate sections processed as above, apart from omitting incubation with the primary antibody solution).
2.5 | Image acquisition and scoring procedure

All images were captured with a Leica DM 6000 microscope using IP-lab imaging software (Scanalytics Inc, Fairfax, VA). Stained sections were viewed at a magnification of ×400 and manually scored by at least two independent researchers according to γ-H2AX staining intensity, where 0 indicates no staining present, 1 for light staining, 2 for moderate staining, and 3 for intense staining. Figure 1 shows examples of the different staining intensities found. Percentage of positive cells scored as follows: 0 for ≤5%, 1 for 6%–25%, 2 for 26%–50%, 3 for 51%–75%, and 4 for ≥76%. Positive was marked if the products of staining intensity and the score of the percentage of positive cells ≥1.

2.6 | Other data

Patients’ clinical and pathological data were collected, including age of onset, tumor size, lymph node metastasis, histological grade, TNM stage, and results of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth receptor 2 (HER2), P53, and Ki-67. Patients’ OS time was calculated from the day of surgery until the death in months.

2.7 | Statistical analyses

Data were analyzed by SPSS 13.0 software (SPSS v13.0; ISPP Inc., Chicago, IL). Continuous data were presented as mean ± standard deviation and categorical data was presented as proportion. The χ² test was used to evaluate any potential association between γ-H2AX expression and the clinicopathological parameters. Patients’ OS time was measured in months from the day of surgery until death. Survival rates were calculated with the Kaplan-Meier method, and the statistical difference between survival curves was determined with the log-rank test. Multivariate Cox proportional hazards regression analysis, using a forward stepwise selection approach, was performed to explore the independent effect of variables on survival. All tests were two-tailed, and statistical significance was considered to be P < 0.05.

3 | RESULTS

A total of 97 patients were included in the study (Table 1). All patients were female, aged between 32 and 87 years. The tumor size in 38 (39.9%) patients were greater than 20 mm. Twenty-nine (34.1) patients had lymph node metastases. Of the 97 patients, 4 (4.0%) patients had intraductal carcinoma, 60 (61.9%) had invasive ductal carcinoma, 30 (30.9%) had infiltrating lobular carcinoma, 2 (2.1%) had mucinous adenocarcinoma, and 2 (2.1%) had signet ring cell carcinoma. Among the patients, there are 75 (77.5%) patients with ER positive, 59 (60.8%) patients with PR positive, 7 (7.2%) patients with HER2 positive, 40 (44.0%) patients with P53 positive, and 74 (82.2%) patients with Ki-67 positive.

![Figure 1](image-url) Differences in γ-H2AX expression in breast tumors. The representative views of γ-H2AX staining from top to bottom are negative (A), weak-positive (B), and positive (C and D). Original magnification: ×400; scale bar, 100 μm
3.1 | Expression of γ-H2AX in breast tumors and adjacent normal tissues

The expression of γ-H2AX was analyzed in the all tumor tissues (97 cases) and adjacent normal tissues (77 cases). In paired tumor tissue and adjacent normal tissues, the positive rate of γ-H2AX in tumor tissue was 26.0% (Table 2), while in adjacent normal tissues, the positive rate was 2.6%. The positive rate of γ-H2AX in tumor tissues was significantly higher than that in adjacent normal tissues (P < 0.001).

3.2 | Association of γ-H2AX with clinicopathologic variables

The γ-H2AX staining was positive in 27 (27.8%) patients. γ-H2AX positive status was associated with earlier TNM staging, with 24 positive cases (32.4%) in TNM staging I-II, but no positive cases in TNM staging III-IV (P = 0.026). There were no significant difference in age at diagnosis, tumor size, lymph node metastasis, histological grade, ER, PR, HER2 status, P53, and Ki-67 results between patients with γ-H2AX positive and γ-H2AX negative (Table 3).

3.3 | Overall survival by γ-H2AX status in patients with breast cancer

Sixteen patients (16.5%) died during the follow-up. The median follow-up for OS in 97 patients with γ-H2AX data was 77 months, ranging from 6 to 93 months. The relation between γ-H2AX and cancer progression was evaluated. In the γ-H2AX positive group, mean OS was 80.1 months (95% CI, 73.3-87.0; Table 4), compared with 83.9 months (95% CI, 78.9-87.0) in those with γ-H2AX negative tumors (P = 0.76; Figure 2). No significant association between γ-H2AX and OS was found. In Cox regression model, γ-H2AX positive was associated with insignificant lower risk (hazard ratio [HR]: 0.84, 95% CI: 0.27, 2.60). In multivariable Cox regression modeling analysis, γ-H2AX status were not significantly associated with outcome. Only age and TNM staging entered the model. The HR for age was 1.08 (95% CI, 1.03-1.14), the HR for TNM III-IV was 4.69 (95% CI, 1.37-16.08).

We observed that γ-H2AX expression is negatively correlated with TNM staging, and TNM staging is significantly associated with survival, so we further examined the relationship between γ-H2AX expression and survival in different TNM staging subgroups. In patients with TNM stage I-II, 6 out of 50 γ-H2AX negative cases died (Table 5), while only 1 deaths were observed in the 24 γ-H2AX positive patients. In patients with TNM stage III-IV, 4 out of 11 γ-H2AX negative patients died. In the subgroup analysis, we observed patient death mostly in γ-H2AX negative patients.


**DISCUSSION**

In this study, we observed a significantly higher positive rate of \( \gamma \)-H2AX in tumor cells than that in adjacent normal tissues. The positive rate of \( \gamma \)-H2AX in tumor cell negatively correlated with TNM staging. In TNM I-II subgroups, mortality in \( \gamma \)-H2AX negative cases was significantly higher. No significant association between \( \gamma \)-H2AX and OS was observed.

The observed higher \( \gamma \)-H2AX positive rate in tumor tissue comparing to adjacent normal tissues is consistent with previous reports in patients with cancer.\(^{13,14}\) Martin et al\(^{13}\) has shown in patients with various solid tumors, the expression of \( \gamma \)-H2AX was significantly higher than that in the adjacent normal tissues. Brunner et al\(^{14}\) showed a much higher expression of \( \gamma \)-H2AX in endometrial carcinomas than adjacent noncancerous epithelial lesions. At molecular level, histone H2AX phosphorylation on serine 139 (\( \gamma \)-H2AX) has been shown to be one of the earliest cellular responses to DNA DSBs formation.\(^{15,16}\) \( \gamma \)-H2AX recruits other factors such as 53BP1, BRCA1, MDC1, and the MRE11-RAD50-NBS1 (MRN) complex to sites of damage to repair the damage.\(^{17-20}\) DNA damage is an upstream event in this concept, H2AX phosphorylation in response to DNA damage is a crucial step in the process of tumorigenesis.\(^{13}\) In our study, we observed a higher expression of \( \gamma \)-H2AX in tumor cells than adjacent normal tissues, which may be indicative of defective DNA repair pathway or genomic instability in tumor cells. The significant difference in tumor and adjacent tissues demonstrates that \( \gamma \)-H2AX might help to improve the efficiency in early diagnosis.

To our knowledge, the observed negative associations of \( \gamma \)-H2AX expression and TNM staging in breast cancer has not been previously reported. Lee et al\(^{21}\) showed

| Characteristics                  | n   | \( \gamma \)-H2AX expression | \( \chi^2 \) | P   |
|----------------------------------|-----|-------------------------------|-------------|-----|
|                                 |     | Negative | Positive |     |     |
| Age at diagnosis                 |     |          |          |     |     |
| \( \leq 60 \)                  | 66  | 70       | 27       | 1.327 | 0.249 |
| \( >60 \)                      | 31  | 50       | 16       | 11   |
| Tumor diameter, mm               |     |          |          |     |     |
| \( \leq 20 \)                  | 59  | 70       | 27       | 1.264 | 0.261 |
| \( >20 \)                      | 38  | 45       | 14       | 13   |
| Lymph node metastasis            |     |          |          |     |     |
| Negative                        | 56  | 61       | 24       | 1.237 | 0.266 |
| Positive                        | 29  | 38       | 18       | 6    |
| Histological grade              |     |          |          |     |     |
| I                               | 15  | 68       | 26       | 0.523 | 0.469 |
| II-III                          | 79  | 56       | 23       | 23   |
| TNM staging                     |     |          |          |     |     |
| I-II                            | 74  | 61       | 24       | 4.971 | 0.026(a) |
| III-IV                          | 11  | 11       | 0        | 0    |
| ER                              |     |          |          |     |     |
| Negative                        | 22  | 70       | 27       | 2.421 | 0.120 |
| Positive                        | 75  | 57       | 18       | 18   |
| PR                              |     |          |          |     |     |
| Negative                        | 38  | 70       | 27       | 0.436 | 0.509 |
| Positive                        | 59  | 26       | 12       | 12   |
| HER2                            |     |          |          |     |     |
| Negative                        | 90  | 64       | 26       | 0.690 | 0.406 |
| Positive                        | 7   | 6        | 1        | 1    |
| P53                             |     |          |          |     |     |
| Negative                        | 51  | 67       | 24       | 0.047 | 0.829 |
| Positive                        | 40  | 38       | 13       | 13   |
| Ki-67                           |     |          |          |     |     |
| Negative                        | 16  | 65       | 25       | 0.917 | 0.338 |
| Positive                        | 74  | 55       | 19       | 19   |

Abbreviations: ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

(a) Fisher’s exact test.
that in patients with colorectal cancer, high expression of γ-H2AX correlated with advanced TNM stage. Xiao et al.\textsuperscript{22} showed in hepatocellular carcinoma that high expression of γ-H2AX is also associated with advanced TNM staging; the authors suggested low γ-H2AX expression levels may be indicative of a slowly proliferating, less aggressive tumor phenotype with good prognostic features. But in our study, we observed a negative association of γ-H2AX expression and TNM staging. γ-H2AX helps prevent aberrant repair of both programmed and general DNA breakage, and functions as a dosage-dependent tumor suppressor.\textsuperscript{23} Klokov et al.\textsuperscript{24} found that the percentage of tumor cells retaining γ-H2AX foci after irradiation was a useful measure of cellular radiosensitivity. Taneja et al.\textsuperscript{25} observed that radiosensitive tumor cells and xenografts retained γ-H2AX for a greater duration than radioresistant cells and tumors. Moon et al.\textsuperscript{26} reported that wild-type p53-induced phosphatase 1 could significantly reduce the level of H2AX, and the forced premature dephosphorylation could disrupt recruitment of important DNA repair factors to damaged sites and delay DNA damage repair. It is possible in advanced TNM stages that γ-H2AX was cleared faster because γ-H2AX degradation or dephosphorylation, thus resulting in lower DSBs repairs. The lower γ-H2AX positive in advanced TNM staging might suggest a more malignant phenotype. What is more, detecting the expression of γ-H2AX may become an index for the screening of malignancy of breast cancer, which is of guiding significance in the clinical diagnosis and treatment of breast cancer.

**TABLE 4** Univariate log-rank regression survival analyses

| Characteristics          | Cases (mortality) | Survival rate (%) | P     | Mean survival time (95% CI), mo |
|--------------------------|-------------------|-------------------|-------|---------------------------------|
| γ-H2AX                   | 97 (16)           | 83.5              | 0.76  | 84.2 (80.2, 88.4)               |
| Negative                 | 70 (12)           | 82.9              |       | 83.7 (78.9, 88.8)               |
| Positive                 | 27 (4)            | 85.2              |       | 80.1 (73.3, 87.0)               |
| Age at diagnosis, y      |                   |                   |       |                                 |
| ≤ 60                     | 97 (16)           | 83.5              | 0.03  | 84.2 (80.2, 88.4)               |
| > 60                     | 66 (6)            | 90.1              |       | 87.9 (83.9, 91.9)               |
| Tumor diameter, mm       |                   |                   | 0.65  |                                 |
| ≤ 20                     | 97 (16)           | 83.5              |       | 84.1 (79.9, 88.3)               |
| > 20                     | 31 (10)           | 67.7              |       | 75.8 (66.5, 85.2)               |
| Lymph node metastasis    |                   |                   | 0.006 |                                 |
| Negative                 | 85 (11)           | 87.1              |       | 84.2 (80.0, 88.4)               |
| Positive                 | 56 (7)            | 87.5              |       | 87.8 (84.2, 91.4)               |
| Histologic grade         |                   |                   | 0.059 |                                 |
| 1                        | 94 (16)           | 83.5              |       | 84.0 (79.8, 88.3)               |
| 2-3                      | 79 (11)           | 86.1              |       | 86.5 (82.7, 90.2)               |
| 2                        | 15 (5)            | 66.7              |       | 71.7 (55.8, 87.6)               |
| Tumor diameter, mm       |                   |                   | 0.005 |                                 |
| ≤ 20                     | 85 (11)           | 87.1              |       | 86.4 (82.6, 90.2)               |
| > 20                     | 74 (7)            | 90.5              |       | 89.0 (86.3, 91.8)               |
| Lymph node metastasis    |                   |                   |       |                                 |
| Negative                 | 11 (4)            | 63.6              |       | 68.2 (49.1, 87.4)               |
| Positive                 | 15 (5)            | 66.7              |       | 71.7 (55.8, 87.6)               |
| ER                       |                   |                   | 0.87  |                                 |
| Negative                 | 97 (16)           | 83.5              |       | 84.2 (80.0, 88.4)               |
| Positive                 | 22 (3)            | 86.4              |       | 82.0 (70.8, 93.2)               |
| Histologic grade         |                   |                   |       |                                 |
| 1                        | 75 (15)           | 82.7              |       | 84.9 (80.7, 89.1)               |
| 2-3                      |                   |                   |       |                                 |
| Age at diagnosis, y      |                   |                   |       |                                 |
| ≤ 60                     |                   |                   |       |                                 |
| > 60                     |                   |                   |       |                                 |
| Tumor diameter, mm       |                   |                   |       |                                 |
| ≤ 20                     |                   |                   |       |                                 |
| > 20                     |                   |                   |       |                                 |
| Lymph node metastasis    |                   |                   |       |                                 |
| Negative                 |                   |                   |       |                                 |
| Positive                 |                   |                   |       |                                 |
| Histologic grade         |                   |                   |       |                                 |
| 1                        |                   |                   |       |                                 |
| 2-3                      |                   |                   |       |                                 |
| TNM staging              |                   |                   |       |                                 |
| I-II                     |                   |                   |       |                                 |
| III-IV                   |                   |                   |       |                                 |
| ER                       |                   |                   |       |                                 |
| Negative                 |                   |                   |       |                                 |
| Positive                 |                   |                   |       |                                 |
| Abbreviation: ER, estrogen receptor.
cancer survival. Nagelkerke et al\textsuperscript{10} found that in a cohort of patients with triple-negative breast cancer followed at least 5 years, high number of $\gamma$H2AX foci indicated a significantly worse prognosis.\textsuperscript{27} In our study in the general breast cancer population, we did not observe a significant association between $\gamma$H2AX and patients’ survival. Second, our study was a single center study, and the study population may be selective. For example, it is possible that patients with $\gamma$H2AX die faster and local physicians are not able to refer them to our hospital, and this group of patients were not able to participate in our study. The survival bias may explain the discrepancy between our results and previous studies in other cancer populations which linked $\gamma$H2AX with worse outcomes.

It should be noted that our study has several limitations. First, the sample size in this study is small, which may result in the nonsignificant association between $\gamma$H2AX and patients’ survival. Second, our study was a single center study, and the study population may be selective. For example, it is possible that patients with $\gamma$H2AX die faster and local physicians are not able to refer them to our hospital, and this group of patients were not able to participate in our study. The survival bias may explain the discrepancy between our results and previous studies in other cancer populations which linked $\gamma$H2AX with worse outcomes.

Current cancer biomarker studies mostly focused on tumor-specific antigens. Recent reviews suggest using markers of cell proliferation, oncogene-induced senescence, telomerase, DNA damage repair, and corresponding epigenetic markers as general cancer biomarkers, which are powerful and promising for cancer prediction, prognosis, therapeutics, and possibly cancer prevention.\textsuperscript{28-31} DNA damage is an upstream event in this concept of H2AX phosphorylation in response to DNA damage is a crucial step in the process of tumorigenesis. This makes detection of $\gamma$H2AX an attractive biomarker that could serve as an early cancer indicator.\textsuperscript{13} $\gamma$H2AX is already coming to clinics as a therapeutic target for monitoring computed tomography and radiation therapy.\textsuperscript{32,33} Yang et al suggested that $\gamma$H2AX is a prognostic factor in patients who received systemic chemotherapy and data on $\gamma$H2AX can be used in the design of clinical trials to test $\gamma$H2AX and the effects of treatment in breast cancer as well as in many other cancer types.\textsuperscript{8} We have shown that $\gamma$H2AX expression is different in tumor cells and adjacent normal tissues, and is associated with TNM staging in the general breast cancer patients. All support $\gamma$H2AX could be a potential biomarker for cancer diagnostics and prediction. Further validation with larger cohort and longer follow-up time is in need. It is important to include $\gamma$H2AX in new cancer biomarkers for validation and proper evaluation of its impact on cancer surveillance.

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### Conflict of Interests

The authors declare that there are no conflict of interests.

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