Expression of DNA Damage Response Molecules PARP1, γH2AX, BRCA1, and BRCA2 Predicts Poor Survival of Breast Carcinoma Patients

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Abstract

BACKGROUND: Poly(ADP-ribose) polymerase 1 (PARP1), γH2AX, BRCA1, and BRCA2 are conventional molecular indicators of DNA damage in cells and are often overexpressed in various cancers. In this study, we aimed, using immunohistochemical detection, whether the co-expression of PARP1, γH2AX, BRCA1, and BRCA2 in breast carcinoma (BCA) tissue can provide more reliable prediction of survival of BCA patients.

MATERIALS AND METHODS: We investigated immunohistochemical expression and prognostic significance of the expression of PARP1, γH2AX, BRCA1, and BRCA2 in breast carcinoma. The samples were obtained from 90 breast carcinoma patients. Immunohistochemical expression of PARP1, γH2AX, BRCA1, and BRCA2 was examined using the Envision + system. The expression level was assessed using a scoring system. The correlation between expression and survival was analyzed using the Kaplan-Meier method and the log-rank test. The expression of PARP1, γH2AX, BRCA1, and BRCA2 was significantly associated with survival. The expression of PARP1, γH2AX, BRCA1, and BRCA2 was also associated with survival in a multivariate analysis. These findings suggest that the co-expression of PARP1, γH2AX, BRCA1, and BRCA2 in breast carcinoma can provide more reliable prediction of survival of BCA patients.

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PARP1, γH2AX, BRCA1, and BRCA2 in 192 cases of BCAs. RESULTS: The expression of these four molecules predicted earlier distant metastatic relapse, shorter overall survival (OS), and relapse-free survival (RFS) by univariate analysis. Multivariate analysis revealed the expression of PARP1, γH2AX, and BRCA2 as independent poor prognostic indicators of OS and RFS. In addition, the combined expression pattern of BRCA1, BRCA2, PARP1, and γH2AX (CSbbph) was an additional independent prognostic predictor for OS ($P < .001$) and RFS ($P < .001$). The 10-year OS rate was 95% in the CSbbph-low (CSbbph scores 0 and 1) subgroup, but that was only 35% in the CSbbph-high (CSbbph score 4) subgroup. CONCLUSION: This study has demonstrated that the individual and combined expression patterns of PARP1, γH2AX, BRCA1, and BRCA2 could be helpful in determining an accurate prognosis for BCA patients and for the selection of BCA patients who could potentially benefit from anti-PARP1 therapy with a combination of genotoxic chemotherapeutic agents.

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Introduction

Poly(ADP-ribose) polymerase 1 (PARP1) is important in the repair of DNA damage as it immediately binds to DNA breaks to induce recruitment and activation of other DNA repair proteins [1,2]. However, the major role of PARP1 in the repair of DNA single-strand breaks could induce progression of human malignant tumors [3]. The aberrant DNA repairing activity from the overexpression of PARP1 in tumor cells could enhance the anti-apoptotic property of tumor cells, which results in chemotherapy-resistant cancers [3]. Therefore, it is suggested that PARP1 could affect tumor development, and the overexpression of PARP1 is associated with advanced clinical characteristics and poor survival of human malignant tumors, including breast carcinoma (BCA) [4,5], ovarian carcinoma [6], melanoma [7], and glioblastoma [8]. Thus, the antitumoral effect of PARP1 inhibition by small interfering RNA or chemicals has been evaluated, and PARP1 inhibition increased apoptosis of cancer cells when used in conjunction with a DNA damaging therapy [9–11]. In addition, several PARP inhibitors have been developed and are in clinical trials in combination with chemotherapeutic drugs [3,12,13].

γH2AX is the phosphorylated form (serine 139) of the H2AX protein and is important in the repair of DNA double-strand breaks (DSBs) [14–16]. Phosphorylation of H2AX causes a conformational change in the DNA-H2AX complex, which allows room for the recruitment of proteins needed to repair DSBs [17–20]. Therefore, γH2AX levels could increase in conjunction with increases in cancer-associated genomic instability [14]. Consequently, as the expression of PARP1 increases in advanced cancers, increased γH2AX levels may reflect the progression of human cancer. In triple-negative BCA [21] and endometrial cancer [22,23], the expression of γH2AX is associated with poor survival of cancer patients. However, other DNA damage response (DDR) molecules, especially BRCA1/2, are necessary for the repair of DSB. Therefore, if there is no γH2AX-BRCA1/2–related repair for DSB, PARP1 inhibitors eventually induce unreparable DSB. Thus, PARP1 inhibitors could selectively target cancer cells with defects or loss of BRCA1/2[3,24]. Recent reports have shown that PARP inhibitors are effective for the treatment of BRCA-deficient BCA [13,25], but they have had limited success with cancers not associated with BRCA1/2[1,26]. However, the PARP inhibitor, olaparib, was effective in both ovarian carcinomas with a BRCA1/2 mutation and without a BRCA1/2 mutation [27].

Materials and Methods

Patients and Tissue Samples

The BCAs diagnosed between January 1997 and December 2003 in Chonbuk National University Hospital were subjected to this study. Thereafter, 192 cases with original histologic slides, paraffin-embedded tissue blocks, and clinical information available were included in the present study. This study was approved by the Institutional Review Board of Chonbuk National University Hospital. Informed consent was provided according to the Declaration of Helsinki.

The age of the 192 BCA patients ranged from 22 to 73 years (mean, 47 years). The type of operation in 112 patients was modified radical mastectomy, and 80 patients received breast conserving surgery. Postoperatively, 169 patients received systemic chemotherapy (cyclophosphamide, methotrexate, and 5-fluorouracil chemotherapy or anthracycline- and taxane-based chemotherapy), and 166 patients received endocrine therapy. One hundred forty-six patients received both adjuvant chemotherapy and endocrine therapy, and three patients received no adjuvant therapy. The median duration of follow-up was 134.8 months (range, 7.7–198.6). Among the 192 BCA patients, 59 patients experienced relapse and 55 patients died from BCA at the follow-up endpoint. The overall survival (OS) rates at 5 and 10 years were 82% and 75%, respectively. The histologic findings were reviewed and classified according to the World Health Organization Classification [29] by two pathologists (K.Y.J. and S.J.N.). The stage of the BCA was assigned according to the seventh
Immunohistochemical Staining and Scoring

Immunohistochemical expression of PARP1, γH2AX, BRCA1, and BRCA2 was evaluated by established tissue microarray (TMA). The TMAs were arrayed from the original paraffin-embedded tissue blocks at the most representative area composed mainly of tumor cells and have the highest tumor grade. Two 3.0-mm tumor cores were arrayed per case. For the antigen retrieval, the TMA sections were boiled with Dako Target Retrieval Solution (pH 6.0; Dako, Glostrup, Denmark) using a microwave oven for 20 minutes. Thereafter, the TMA sections were incubated with anti-PARP1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), anti-γH2AX (Ser 139; 1:100; Cell Signaling Technology, Beverly, MA), anti-BRCA1 (1:100; Abcam, Cambridge, MA), and anti-BRCA2 (1:100; Abcam) antibodies. The scoring for the immunohistochemical staining was performed by two pathologists (K.Y.J. and K.M.K.) by consensus under a multiviewing microscope without knowledge of the clinicopathologic information. Immunohistochemical staining for PARP1, BRCA1, and BRCA2 was evaluated by the sum of the staining intensity scores (0, no staining; 1, weak staining; 2, intermediate staining; and 3, strong staining) and the staining area scores (0, no staining cells; 1, 1% of the cells stained positive; 2, 2-10% of the cells stained positive; 3, 11-33% of the cells stained positive; 4, 34-66% of the cells stained positive; and 5, 67-100% of the cells stained positive) in each TMA core [31–33]. Thereafter, the scores of two TMA cores from the same case were added and used for the analysis. The sum score ranged from 0 to 16. To quantify the number of γH2AX-positive tumor cells, the number of γH2AX-positive tumor cells was counted in five high-power fields (HPFs; magnification, ×400) in each TMA core at the highest γH2AX-positive numbered area. Thereafter, we added the number of γH2AX-positive tumor cells from the two different TMA cores and used them for the final analysis [34,35]. The diameter of the HPF was 0.55 mm, and the area of one HPF was 0.238 mm². Human epidermal growth factor receptor 2 (HER2) was considered positive when 10% or more of the tumor cells showed complete and intense staining at the cell membrane (3 + by American Society of Clinical Oncology/College of American Pathologists guidelines) [36]. Estrogen receptor (ER) and progesterone receptor (PR) were considered positive when 1% or more of the tumor cells show nuclear expression.

Cell Lines and Western Blot Analysis

MCF7 and MDA-MB-231 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells (5 × 10⁵) were seeded in each well of a six-well plate and incubated at 37°C in a humidified incubator containing 5% CO₂ overnight. Then, cells were treated with 0.1 μM camptothecin in DMSO or DMSO as control. After 30 minutes, cells were harvested for Western blot analysis. The primary antibodies for PARP1 (Santa Cruz Biotechnology), γH2AX (Ser 139) (Cell Signaling Technology), BRCA1 (Abcam), BRCA2 (Abcam), and actin (Santa Cruz Biotechnology) were used in the Western blot analysis.

Statistical Analysis

The BCAs were grouped as positive or negative for the expression of PARP1, γH2AX, or BRCA1 at the specific cutoff points of the immunohistochemical staining scores. The cutoff points were determined by receiver operating characteristic curve analysis at the highest positive likelihood point for the estimation of death. The relationships between the clinicopathologic variables included in this study were determined using Pearson’s chi-square test, and the P values were adjusted by the Benjamini Hochberg procedure for multiple comparison. The prognosis of BCA was evaluated by the analysis of the OS and relapse-free survival (RFS). The endpoint of follow-up was the date of death of patients or the date of last contact through June 2013. The duration of the OS was calculated as the time from the date of diagnosis to date of death from BCA. If the patients were alive at last contact or died from other causes, they were treated as censored. RFS duration was measured as the time from the date of diagnosis to the date of death from BCA, date of relapse, or last contact. Patients who were alive at last contact with no relapse or who died from other causes were treated as censored for RFS analysis. Survival analysis was performed with univariate and multivariate Cox regression hazard analyses and Kaplan-Meier survival analysis with a log-rank test using SPSS statistical software (version 19.0; IBM, Chicago, IL). P values less than .05 were considered to be statistically significant.

Results

The Expression of PARP1, γH2AX, BRCA1, and BRCA2 and Their Association With Clinicopathologic Variables

To validate the antibodies used in this study, we performed Western blot analysis for BRCA1, BRCA2, PARP1, and γH2AX in two BCA cell lines treated with camptothecin, one of the conventional DNA damaging agents. As shown in Figure 1A, these antibodies detected each protein in the expected position and the expression levels of these proteins were upregulated by the treatment of camptothecin. In immunohistochemical staining of BCA tissue, PARP1 and γH2AX are mainly expressed in the nuclei of the tumor cells (Figure 1B). Although BRCA1 and BRCA2 are expressed in both the cytoplasm and nuclei of the tumor cells, we used nuclear expression in this study [28,37]. The cutoff points for the immunohistochemical staining score for PARP1, BRCA1, and BRCA2 were 13, 7, and 9, respectively. The cutoff number of γH2AX-positive tumor cells was 8 (Figure 1C). The expression of PARP1, γH2AX, BRCA1, or BRCA2 was grouped positive in 41% (78/192 of cases), 51% (98/192), 75% (144/192), and 55% (105/192) of BCA, respectively. PARP1 positivity was significantly associated with the development of latent distant metastasis, increased mitotic count, histologic grade, and the expression of BRCA1 and BRCA2. γH2AX positivity was significantly correlated with the development of latent distant metastasis, increased mitotic count, histologic grade, and the loss of ER expression or PR expression. There was an especially strong positive correlation between the expression of PARP1 and γH2AX (P = .004). The number of γH2AX-positive cells was significantly higher in the PARP1-positive group compared with the PARP1-negative group (mean ± standard error, 58 ± 17 vs 26 ± 5, two-sided t test; P = .039). The expression of both BRCA1 and BRCA2 was significantly correlated with the development of latent distant metastasis and higher histologic grade (Table 1).

The Expression of PARP1, γH2AX, BRCA1, and BRCA2 Was Associated With Shorter Survival of BCA Patients by Univariate Analysis

In 192 BCAs, the factors significantly associated with both OS and RFS by univariate survival analyses were the age of the patients, tumor stage, histologic grade, HER2 expression, PR expression, BRCA1 expression (OS, P = .012; RFS, P = .011), BRCA2 expression (OS,
PARP1, γH2AX, BRCA1, and BRCA2 in breast carcinoma

The patients with tumors expressing PARP1 had a 5.778-fold [95% confidence interval (CI), 3.143-10.623] greater risk of death ($P < .001$), and its expression was significantly associated with shorter RFS ($P < .001$; hazard ratio (HR), 3.039; 95% CI, 1.889-4.888). The expression of γH2AX predicted shorter OS ($P < .001$; HR, 4.725; 95% CI, 2.439-9.154) and RFS ($P < .001$; HR, 3.706; 95% CI, 2.172-6.325). The expression of BRCA1 predicted shorter OS ($P = .012$; HR, 2.965; 95% CI, 1.269-6.926)
and RFS \( (P = .011; \text{HR}, 2.392; 95\% \text{CI}, 1.226-4.667) \). The expression of BRCA2 predicted shorter OS \( (P < .001; \text{HR}, 4.284; 95\% \text{CI}, 2.158-8.505) \) and RFS \( (P < .001; \text{HR}, 2.886; 95\% \text{CI}, 1.692-4.925; \text{Table 2}) \).

Thereafter, we did further survival analysis of the subpopulation of BCA patients who received adjuvant chemotherapy or endocrine therapy. Among the 169 BCA patients who received systemic adjuvant chemotherapy, the expression of HER2, PR, BRCA1 \( \log \)-rank, OS, \( P = .001 \); RFS, \( P = .009 \)), BRCA2 \( \log \)-rank, OS, \( P < .001 \); RFS, \( P < .001 \)), PARP1 \( \log \)-rank, OS, \( P < .001 \); RFS, \( P < .001 \)), and \( \gamma \text{H2AX} \) \( \log \)-rank, OS, \( P < .001 \); RFS, \( P < .001 \)) was significantly associated with shorter OS and RFS \( \text{Figure 2A}) \). Older age of the patients and higher tumor stage were associated with shorter OS. Among the 166 BCA patients who received postoperative

**Table 1.** Association of the Expression of PARP1, \( \gamma \text{H2AX}, \text{BRCA1}, \) and \( \text{BRCA2} \) with Clinicopathologic Factors

| Characteristics | PARP1 | \( \gamma \text{H2AX} \) | BRCA1 | \( \gamma \text{H2AX} \) | BRCA2 |
|-----------------|-------|-----------------|-------|-----------------|-------|
| \( P_{\text{BH}} \) |       |                 |       |                 |       |
| Age, years      |       |                 |       |                 |       |
| \(<5\)          | 131   | 48 (37\%)       | .145  | 63 (48\%)       | .336  |
| \(\geq5\)       | 61    | 30 (49\%)       |       | 35 (57\%)       |       |
| TNM stage       |       |                 |       |                 |       |
| I               | 35    | 13 (37\%)       | .581  | 15 (43\%)       | .625  |
| II              | 124   | 49 (40\%)       |       | 67 (54\%)       |       |
| III and IV      | 33    | 16 (48\%)       |       | 16 (48\%)       |       |
| T stage         |       |                 |       |                 |       |
| 1               | 55    | 23 (42\%)       | .145  | 26 (47\%)       | .761  |
| 2               | 122   | 45 (37\%)       |       | 63 (52\%)       |       |
| 3 and 4         | 15    | 10 (67\%)       |       | 9 (60\%)        |       |
| LN metastasis   |       |                 |       |                 |       |
| Absence         | 102   | 36 (35\%)       | .145  | 51 (50\%)       | .810  |
| Presence        | 90    | 42 (47\%)       |       | 47 (52\%)       |       |
| Latent distant metastasis | 148 | 46 (31\%) | <.001 | 64 (43\%) | <.001 |
| Histologic type |       |                 |       |                 |       |
| NST             | 184   | 76 (41\%)       | .382  | 93 (51\%)       | .625  |
| Lobular         | 8     | 2 (25\%)        |       | 5 (63\%)        |       |
| Tubule formation| >75%  | 33 (10\%)       | .256  | 14 (42\%)       | .052  |
| <10\%           | 78    | 37 (47\%)       |       | 49 (63\%)       |       |
| Nuclear pleomorphism | 1  | 17   | 3 (18\%) | .073 | 7 (41\%) | .144 |
| 2               | 92    | 34 (57\%)       |       | 41 (45\%)       |       |
| 3               | 83    | 41 (49\%)       |       | 50 (60\%)       |       |
| Mitoses/10 HPFs | 0-9   | 112  | 36 (32\%) | .100 | 47 (42\%) | .016 |
| >9              | 19    | 42 (44\%)       |       | 24 (57\%)       |       |
| Histologic grade|       |                 |       |                 |       |
| 1               | 65    | 19 (29\%)       | .013  | 25 (38\%)       | .003  |
| 2               | 88    | 55 (40\%)       |       | 43 (49\%)       |       |
| 3               | 39    | 24 (62\%)       |       | 30 (77\%)       |       |
| HER2            |       |                 |       |                 |       |
| Negative        | 128   | 49 (38\%)       | .382  | 60 (47\%)       | .163  |
| Positive        | 64    | 29 (45\%)       |       | 38 (59\%)       |       |
| ER              |       |                 |       |                 |       |
| Negative        | 86    | 42 (49\%)       | .074  | 58 (67\%)       | <.001 |
| Positive        | 106   | 56 (34\%)       |       | 40 (38\%)       |       |
| PR              |       |                 |       |                 |       |
| Negative        | 92    | 43 (47\%)       | .145  | 55 (60\%)       | .046  |
| Positive        | 100   | 35 (35\%)       |       | 43 (43\%)       |       |
| BRCA2           |       |                 |       |                 |       |
| Negative        | 87    | 13 (15\%)       | <.001 | 36 (41\%)       | .040  |
| Positive        | 105   | 65 (62\%)       |       | 62 (59%)        |       |
| BRCA1           |       |                 |       |                 |       |
| Negative        | 48    | 4 (8\%)         | <.001 | 24 (50\%)       | .868  |
| Positive        | 144   | 74 (51\%)       |       | 74 (51\%)       |       |
| \( \gamma \text{H2AX} \)           |       |                 |       |                 |       |
| Negative        | 94    | 27 (29\%)       | .004  |                   |       |
| Positive        | 98    | 51 (52\%)       |       |                   |       |
| PARP1           |       |                 |       |                 |       |
| Negative        | 114   |                   | .039† |                   |       |
| Positive        | 78    |                   | .087  |                   |       |

Abbreviations: LN, lymph node; NST, invasive carcinoma of no special type; \( P_{\text{BH}} \), chi-square test adjusted by Benjamini-Hochberg method.

* The mean number of \( \gamma \text{H2AX}-positive \) cells ± standard error.
† Two-sided t test.

**Figure 1.** The expression and prognostic significance of PARP1, \( \gamma \text{H2AX}, \text{BRCA1}, \) and \( \text{BRCA2} \) in 192 BCAs. (A) Validation of the antibodies used in this study. Two breast cancer cell lines (MCF7 and MDA-MB-231) were treated with camptothecin \( (0.1 \mu \text{M}) \) for 0.5 hour and lysed for Western blot analysis of BRCA1, \( \gamma \text{H2AX}, \text{PARP1}, \) and \( \gamma \text{H2AX} \) expression. The treatment of camptothecin increased the expressions of PARP1, \( \gamma \text{H2AX}, \text{BRCA1}, \) and \( \text{BRCA2} \). (B) Immunohistochemical expression of PARP1, \( \gamma \text{H2AX}, \text{BRCA1}, \) and \( \text{BRCA2} \) in BCA. Original magnification, \( \times400 \). (C) The receiver operating characteristic curve analysis for the determination of cutoff points for the immunohistochemical staining scores of PARP1, \( \gamma \text{H2AX}, \text{BRCA1}, \) and \( \text{BRCA2} \). The cutoff points were determined at the highest area under the curve value representing the highest positive likelihood point for the estimation of the death of patients. The arrowhead indicates the cutoff point for PARP1 immunostaining, and the arrow indicates the cutoff point for the number of \( \gamma \text{H2AX}-positive \) tumor cells. The empty arrowhead indicates the cutoff point for BRCA1 immunostaining, and the empty arrow indicates the cutoff point for the number of \( \text{BRCA2} \) immunostaining. Cases with scores equal or greater than 13 for PARP1 expression were considered positive. The expression of \( \gamma \text{H2AX} \) was considered positive when the number of \( \gamma \text{H2AX}-positive \) cells was equal or greater than eight. The expression of BRCA1 was considered positive when the scores were equal or greater than 7. The expression of \( \text{BRCA2} \) was considered positive when the scores were equal or greater than 9. (D) Kaplan-Meier survival analysis for the OS and RFS according to the expression of PARP1, \( \gamma \text{H2AX}, \) and \( \text{BRCA1} \).
endocrine therapy, the age, histologic grade, and the expression of HER2, PR, BRCA1 (log-rank, OS, \( P = .009 \); RFS, \( P = .009 \)), BRCA2 (log-rank, OS, \( P < .001 \); RFS, \( P < .001 \)), PARP1 (log-rank, OS, \( P < .001 \); RFS, \( P < .001 \)), and H2AX (log-rank, OS, \( P < .001 \); RFS, \( P < .001 \)) were significantly associated with both OS and RFS (Figure 2A).

Among the 33 triple-negative BCAs (HER2/ER-/PR-), PARP1 expression predicted shorter OS (\( P = .017 \); HR, 12.256; 96% CI, 1.564-96.035) and RFS (\( P = .046 \); HR, 3.227; 96% CI, 1.023-10.172). H2AX positivity was significantly associated with shorter OS (log-rank, \( P = .002 \)) and RFS (\( P = .015 \); HR, 6.389; 95% CI, 1.433-28.486). BRCA2 expression was significantly associated with shorter OS (\( P = .018 \); HR, 6.429; 95% CI, 1.382-29.909). However, the expression of BRCA1 was not associated with the prognosis of the triple-negative BCA (Figure 2C).

Furthermore, we evaluated the prognostic effect of the combined expression of PARP1, H2AX, BRCA1, and BRCA2. When we focused our analysis on the expression status of BRCA1 and BRCA2, PARP1 expression predicted shorter OS and RFS in the BRCA1+, BRCA1+, BRCA2+, and BRCA2+ subgroups (Table 3). PARP1 expression also predicted shorter OS in the both H2AX- and H2AX+ subgroups (Table 3).\( H2AX \) positivity was associated with shorter OS and RFS in the BRCA1+, BRCA1+, BRCA2+, and PARP1+ subgroups (Table 3). Because the expressions of PARP1, \( H2AX \), BRCA1, and BRCA2 were closely related (Table 1), the combined score for the immunohistochemical expression of BRCA1, BRCA2, PARP1, and \( H2AX \) (CSbbph) was established with the sum of positivity of BRCA1, BRCA2, PARP1, and \( H2AX \) (negative, 0; positive, 1; \( H2AX = 1 + 1 + 1 \) = CSbbph 4). The CSbbph ranged from zero (BRCA1+/BRCA2-/PARP1+/H2AX+) to four (BRCA1+/BRCA2+/PARP1+/H2AX+). Thereafter, CSbbph scores were grouped as CSbbph-low (CSbbph 0-1), CSbbph-intermediate (CSbbph 2-3), and CSbbph-high (CSbbph 4). Among the 192 general cases of BCA, CSbbph was significantly associated with OS (\( P < .001 \)) and RFS (\( P < .001 \); Figure 3 and Table 2). The OS rates at 10 years (10y-OS) of the CSbbph-low, the CSbbph-intermediate, and the CSbbph-high subgroups were 95%, 79%, and 35%, respectively (Figure 3).

### Table 2. Univariate Cox Proportional Hazards Regression Analysis for OS and RFS in BCA Patients

| Characteristics       | No.    | OS HR (95% CI) | P     | RFS HR (95% CI) | P     |
|-----------------------|--------|---------------|-------|----------------|-------|
| Age, years, ≥ 50 (n ≤ 50) | 61/192 | 2.808 (1.652-4.773) | <.001 | 1.713 (1.072-2.739) | .025  |
| TNM stage             |        |               |       |                |       |
| I                     | 35/192 | 1             | .002  | 1              | .045  |
| II                    | 124/192| 2.691 (0.957-7.573) | .061  | 1.860 (0.879-3.937) | .105  |
| III and IV            | 33/192 | 5.915 (1.974-17.719) | .001  | 2.877 (1.241-6.670) | .014  |
| Histologic grade      |        |               |       |                |       |
| 1                     | 65/192 | 1             | <.001 | 1              | .046  |
| 2                     | 88/192 | 1.482 (0.732-2.979) | .269  | 1.182 (0.674-2.074) | .56   |
| 3                     | 39/192 | 3.527 (1.723-7.222) | .025  | 2.072 (1.138-3.824) | .02   |
| HER2, positive (n negative) | 64/192 | 1.836 (1.079-3.123) | .021  | 1.608 (1.006-2.569) | .047  |
| ER, negative (n positive) | 86/192 | 1.813 (1.063-3.091) | .029  | 1.475 (0.929-2.343) | .099  |
| PR, negative (n positive) | 92/192 | 2.125 (1.233-3.662) | .007  | 2.066 (1.286-3.320) | .003  |
| BRCA2, positive (n negative) | 105/192 | 4.284 (2.158-8.509) | <.001 | 2.886 (1.692-4.923) | <.001 |
| BRCA1, positive (n negative) | 144/192 | 2.965 (1.269-6.926) | <.001 | 2.392 (1.226-4.667) | <.001 |
| PARP1, positive (n negative) | 98/192 | 5.778 (3.143-10.623) | <.001 | 3.039 (1.889-4.888) | <.001 |
| γH2AX, positive (n negative) | 78/192 | 4.725 (2.439-9.154) | <.001 | 3.706 (2.172-6.325) | <.001 |
| CSbbph                 |        |               |       |                |       |
| Low                   | 68/192 | 1             | <.001 | 1              | <.001 |
| Intermediate          | 81/192 | 4.535 (1.556-13.212) | .006  | 2.789 (1.367-5.689) | .005  |
| High                  | 43/192 | 18.805 (6.608-53.519) | <.001 | 7.975 (3.894-16.336) | <.001 |
Figure 2. Kaplan-Meier survival analysis according to the expression of PARP1, γH2AX, BRCA1, and BRCA2 in the subpopulations of BCAs. (A) OS and RFS in 169 BCA patients who received adjuvant chemotherapy. (B) OS and RFS in 166 BCA patients who received postoperative endocrine therapy. (C) OS and RFS in 33 triple-negative (HER2⁻/ER⁻/PR⁻) BCA patients.
malignant tumors [4–8,21,22]. PARP1 is involved in the chemoresistance, and c-Myc–bridging integrator 1 (BIN1)–PARP1 signaling pathways induce resistance to cisplatin; overexpression of c-Myc suppresses BIN1 and consequently releases PARP1, resulting in an induction of chemoresistance [39]. In addition, the inhibition of PARP1 activity induced BIN1-mediated suppression of c-Myc [39].

Table 3. Univariate Cox Proportional Hazards Regression Analysis for Survival in Various Subgroups of BCA according to the Expression of BRCA1, BRCA2, PARP1, and γH2AX

| IHC Subgroup | No. | PARP1, Positive (vs Negative) | γH2AX, Positive (vs Negative) | BRCA2, Positive (vs Negative) | BRCA1, Positive (vs Negative) |
|--------------|-----|------------------------------|------------------------------|-------------------------------|-------------------------------|
|              | OS  | RFS | OS  | RFS | OS  | RFS | OS  | RFS | OS  | RFS |
| BRCA1 Negative 48 | HR (95% CI) | 7.581 (1.364-42.120) | 5.890 (1.516-22.875) | 5.393 (0.629-46.224) | 2.694 (0.696-10.426) | 4.710 (0.853-25.996) | 1.986 (0.421-9.361) |
| Positive 144 | HR (95% CI) | 5.106 (2.539-10.266) | 2.422 (1.429-4.104) | 4.775 (2.381-9.577) | 3.995 (2.229-7.159) | 3.64 (1.548-8.557) | 0.003 |
| P | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 |
| BRCA2 Negative 87 | HR (95% CI) | 6.456 (1.866-22.328) | 3.237 (1.214-8.633) | 3.422 (0.884-13.241) | 2.431 (0.942-6.275) | 1.447 (0.407-5.144) | 1.289 |
| Positive 105 | HR (95% CI) | 3.563 (1.710-7.426) | 2.026 (1.128-3.639) | 4.465 (2.076-9.603) | 3.949 (2.032-7.676) | 0.842 (0.280-4.774) | 1.893 |
| P | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 |
| γH2AX Negative 94 | HR (95% CI) | 3.409 (1.033-11.248) | 2.104 (0.830-5.335) | 3.155 (0.837-11.894) | 1.891 (0.733-4.880) | 0.836 (0.452-27.687) | 1.931 |
| Positive 98 | HR (95% CI) | 5.353 (2.565-11.172) | 2.706 (1.530-4.784) | 3.915 (1.743-8.795) | 2.892 (1.488-5.622) | 1.156 (0.581-2.733) | 1.615 |
| P | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 |
| PARP1 Negative 114 | HR (95% CI) | 2.632 (0.882-7.858) | 2.861 (1.320-6.201) | 3.115 (1.142-9.309) | 2.432 (1.150-5.146) | 1.465 (0.457-4.692) | 1.931 |
| Positive 78 | HR (95% CI) | 4.507 (1.891-10.741) | 3.577 (1.654-7.733) | 3.057 (1.086-8.469) | 2.242 (0.662-3.485) | 0.825 (0.255-2.666) | 0.747 |
| P | <.001 | <.001 | <.001 | <.001 | <.001 | <.001 |
In prostatic cancer, increased resistance to genotoxic reagents in prostate cancer stem–like cells was associated with increased expression of γH2AX that arrests cell cycle in the G2/M phase [40]. Therefore, inhibiting PARP- and/or γH2AX-mediated DNA repair responses during chemotherapy could be a good stratagem for the treatment of subgroup of BCA patients with tumors expressing PARP1 and γH2AX.

When there is no γH2AX-BRCA1/2–related repair of DSB, PARP1 inhibitors block PARP1-mediated repair of the single-stand breaks, resulting in death of tumor cells from unrepairable DSB. Therefore, cancers with BRCA1/2 mutations could be susceptible to treatments with PARP1 inhibitors [3,13,25], and a recent report has shown that the PARP1 inhibitor, olaparib, could be employed in the treatment of BRCA1/2-deficient BCA [13]. Thus, the prognostic implications of PARP1 expression could vary according the BRCA1/2 expression status. However, the expression of PARP1 was a poor prognostic indicator in the general population of BCA [5] and lymph node negative stage II BCA [41]. Our result also showed that the expression of PARP1 is associated with poor prognosis in both BRCA1– and BRCA1+ subgroups. Moreover, the patients with BRCA1+/BRCA2+/PARP1+/γH2AX+ BCA showed the shortest survival with 35% OS at 10 years. These results suggest the possibility that PARP1 inhibitors might be useful for the treatment of BCA patients regardless of the expression status of BRCA1. Although some reports have shown that PARP inhibitors do not show promising results outside of BRCA-associated BCA patients [1,26], the survival benefits of veliparib, a PARP inhibitor, plus temozolomide chemotherapy in metastatic BCAs have been reported [26]. Olaparib, an oral PARP inhibitor, also demonstrated therapeutic effectiveness in the ovarian carcinoma without BRCA1/2 mutation [27]. In addition, the usefulness of PARP inhibitors has been suggested in RECQL4/hormone receptor–deficient tumors and that was independent of BRCA1-ness [42]. In the BCA subgroup receiving adjuvant chemotherapy in our study, the expression of PARP1 and γH2AX was also significantly associated with shorter OS and RFS. Moreover, recently, it has been reported that two kinds of PARP inhibitors, olaparib and rucaparib, potentiated antitumor activity of trastuzumab in HER2-overexpressing BCA [12]. However, our study has the limitation in that we did not investigate the mutation of BRCA1.

Thus, it is not clear whether the immunohistochemical loss of BRCA1 and/or BRCA2 expression could be useful in the estimation of the mutation of the BRCA1/2 gene. In addition, it has been reported that the expression of PARP1 is upregulated in triple-negative BCA [4]. However, in our study, the expression of PARP1 (P = .162) or γH2AX (P = .227) was not significantly different between triple-negative BCA and non–triple-negative BCA. In contrast, as shown in Figure 2C, the expression of PARP1 and γH2AX correlated with shorter survival of triple-negative BCA patients. However, further study is needed to clarify whether the expression of PARP1 and γH2AX really affects the survival of triple-negative BCA patients because of the relatively low number of triple-negative cases in this study. Nevertheless, a recent report showed a reliable correlation between BRCA1 immunostaining and BRCA1 mutation in ovarian carcinomas. Negative or weak staining in less than 10% of tumor cells for BRCA1immunostaining was predictive of BRCA1 mutation [28]. That criterion was similar to the cutoff point for BRCA1 immunostaining used in our study. If 10% of tumor cells stained weakly in two TMA cores, they were scored as six and included in the BRCA1 subgroup. Thereby, on the basis of our cutoff value for the BRCA1 immunostaining, our findings suggest that the prognostic value of PARP1 expression for BCA patients may also be predictive for BCA patients, who have not had a molecular event in BRCA1.

Concerning the prognostic impact of BRCA1 and BRCA2 expression status, our results have shown that the loss of immunohistochemical expression of BRCA1 and BRCA2 is associated with favorable prognosis. However, the prognostic impact of BRCA1/2 expression status has been debated in the literature. Earlier reports showed that BRCA1/2-related BCA had a favorable prognosis [43]; however, poor prognosis in BRCA1/2-mutated BCA patients has also been reported [44] and there were no prognostic differences between BRCA1-related BCA and BRCA1-unrelated BCA in other reports [45,46]. The 10-year survival rates for carriers of the BRCA1 mutation and non-carriers were reported as 80.9% and 82.2%, respectively [46]. However, in our study, immunohistochemical expression of both BRCA1 and BRCA2 was significantly associated with shorter OS and RFS. The 10-year OS rates were 90%, 91%, 70%, and 62% in the BRCA1+–, BRCA2+, BRCA1+-, and BRCA2+ subgroups, respectively. Similarly, a recent report has shown that immunohistochemical expression of nuclear BRCA1 is associated with poor survival of BCA [37] and ovarian serous carcinomas [47]. However, when considering the role of BRCA1/2 as a potent tumor suppressor, the poor prognosis in BRCA1/2-expressing BCA patients is paradoxical. This finding might be related with the fact that BRCA1/2-defective cells are more sensitive to chemotherapeutic agents. In ovarian carcinomas, BRCA1/2 defectiveness was related with platinum resistance [48–50]. In our study, nuclear expression of BRCA1 and BRCA2 was associated with shorter survival in the subgroup of BCA patients who received adjuvant chemotherapy. Therefore, it is suggested that BRCA-ness is associated with chemoresistance. In addition to the nuclear expression of BRCA1, it has been suggested that cytoplasmic expression of BRCA1 is representative of mutant BRCA1 [51]. Therefore, we separately analyzed the cytoplasmic expression of BRCA1/2 and expected that the result might be opposite to the result from the nuclear expression of BRCA1. However, cytoplasmic expression of BRCA1 and BRCA2 was also significantly associated with shorter OS (log-rank, BRCA1, P < .001; BRCA2, P < .001) and RFS (log-rank, BRCA1, P < .001; BRCA2, P < .001; Figure S1). These findings suggest that generalized
expression levels of BRCA1/2 might influence the progression of BCA and/or response to the chemotherapy, but further studies are needed to clarify the role of BRCA1/2 according to its intracellular localization.

Another interesting result of our study is that the combined expression patterns of BRCA1, BRCA2, PARP1 and γH2AX were very predictive of the survival of BCA patients. When three or more markers are included in the negative group (CSbbph-low), the 10y-OS was 95% and that represented 35% (68/192) of BCA patients. The survival rate gradually decreased with the increase of the CSbbph score. The 10y-OS of the CSbbph-intermediate subgroups was 79% and that was only 35% in the CSbbph-high subgroup. This poorest survival group represented 22% (43/192) of BCA patients. Therefore, our results suggest that evaluating the immunohistochemical expression patterns of BRCA1, BRCA2, PARP1, and γH2AX is very helpful for the prediction of the prognosis of BCA patients. In addition, the poor prognostic BCA group expressing DDR molecules could potentially benefit from treatments with drugs such as PARP1 inhibitors, which target DNA damage–related molecules.

In conclusion, this study has shown that the expression of DDR signaling molecules is closely correlated with and helpful for the prediction of the prognosis of BCA patients. Especially, when these DDR signaling molecules are expressed simultaneously, as in the CSbbph-high subgroup in our study, the patients showed very short survival. To the best of our knowledge, this is the first report describing the possible prognostic role of the co-expression of BRCA1, BRCA2, PARP1, and γH2AX in breast cancer patients. Moreover, when we consider that the immunohistochemical evaluation of biopsies is easy and practical, our results suggest that immunohistochemical evaluation of BRCA1, BRCA2, PARP1, and γH2AX could be helpful for the prediction of the prognosis of BCA and for the selection of BCA patients who could potentially be the subject of anti-PARP1 therapy during genotoxic agent-based adjuvant chemotherapy.

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