Translational Oncology
www.transonc.com

Volume 8 Number 4 August 2015 pp. 239–249 239

Expression of DNA Damage Response Molecules PARP1, γH2AX, BRCA1, and BRCA2 Predicts Poor Survival of Breast Carcinoma Patients[1]

See-Hyoung Park*, Sang Jae Noh†, Kyoung Min Kim†, Jun Sang Bae†, Keun Sang Kwon‡, Sung Hoo Jung§, Jung Ryul Kim¶, Ho Lee#, Myoung Ja Chung†, Woo Sung Moon†, Myoung Jae Kang† and Kyu Yun Jang†

*Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, CA, USA; †Department of Pathology, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju, Republic of Korea; ‡Department of Preventive Medicine, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju, Republic of Korea; §Department of Surgery, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju, Republic of Korea; ¶Department of Orthopaedic Surgery, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju, Republic of Korea; #Department of Forensic Medicine, Chonbuk National University Medical School, Research Institute of Clinical Medicine of Chonbuk National University, Biomedical Research Institute of Chonbuk National University Hospital and Research Institute for Endocrine Sciences, Jeonju, Republic of Korea

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

Address all correspondence to: Kyu Yun Jang, MD, PhD, Department of Pathology, Chonbuk National University Medical School, San 2-20 Keumam-dong, Dukjin-gu, Jeonju, 561-180, Republic of Korea.
E-mail: kyjang@chonbuk.ac.kr

[1]This work was supported by a grant (2014) from Chonbuk National University Medical School Fund and by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP; No. 2008-0062279). The biospecimens for this study were provided by the Biobank of Chonbuk National University Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All samples derived from the National Biobank of Korea were obtained with informed consent under Institutional Review Board–approved protocols.
Received 17 February 2015; Revised 18 April 2015; Accepted 24 April 2015

1936-5233/15
http://dx.doi.org/10.1016/j.tranon.2015.04.004

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 expressional 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.

Translational Oncology (2015) 8, 239–249

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-stand 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 unrepairable 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].

When considering the relationships between the expression of PARP1, the phosphorylation of H2AX, and the induction of BRCA1/2, there is a possibility that these molecules are cooperatively involved in the progression of cancer through their roles in the resistance to DNA damaging agents. Moreover, recent reports have shown that evaluation of the expression of these molecules by immunohistochemistry is helpful for the evaluation of the effect of the expression of these molecules [5,21,28]. However, there are no reports that strategically evaluated the expression of these molecules in breast cancer. Therefore, this study investigated the immunohistochemical expression of PARP1, γH2AX, BRCA1, and BRCA2 and evaluated the combined expression of these molecules in the prognosis of BCAs.

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

edition of the American Joint Committee on Cancer staging system [30].

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 mm^2. 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 (5×10^5) were seeded in each well of a six-well plate and incubated at 37°C in a humidified incubator containing 5% CO_2 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.

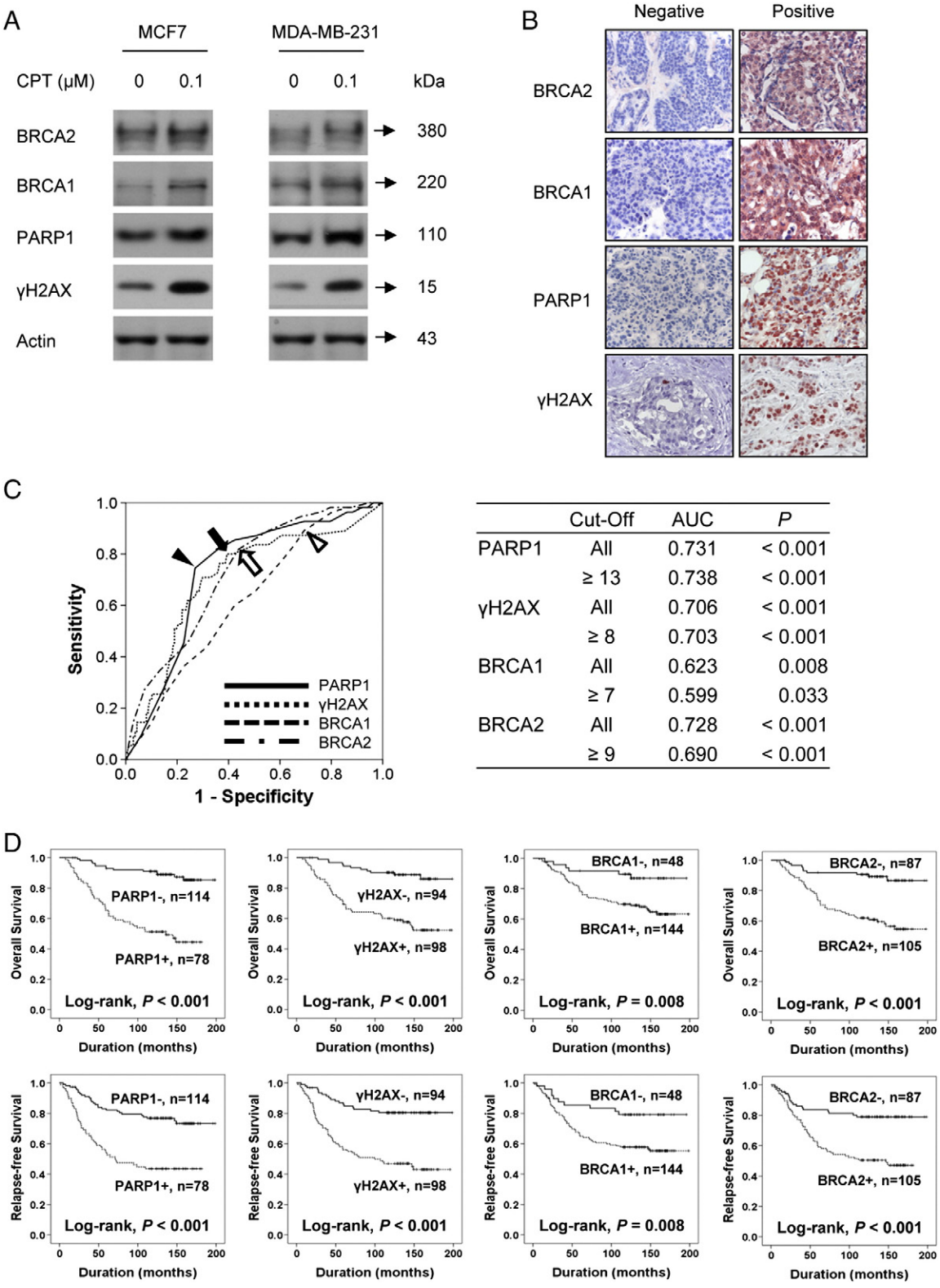
Results

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 1*A*, 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 1*B*). Although BRCA1 and BRCA2 are expressed in both the cytoplasm and nuclei of the tumor cells, we have 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 1*C*). 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,



	Cut-Off	AUC	*P*
PARP1	All	0.731	< 0.001
	≥ 13	0.738	< 0.001
γH2AX	All	0.706	< 0.001
	≥ 8	0.703	< 0.001
BRCA1	All	0.623	0.008
	≥ 7	0.599	0.033
BRCA2	All	0.728	< 0.001
	≥ 9	0.690	< 0.001

P < .001; RFS, *P* < .001), PARP1 expression (OS, *P* < .001; RFS, *P* < .001), and γH2AX positivity (OS, *P* < .001; RFS, *P* < .001; Figure 1*D* and Table 2). 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)

Table 1. Association of the Expression of PARP1, γH2AX, BRCA1, and BRCA2 with Clinicopathologic Factors

Characteristics		No.	PARP1		γH2AX		BRCA1		BRCA2	
			Positive	P_{BH}	Positive	P_{BH}	Positive	P_{BH}	Positive	P_{BH}
Age, years	<50	131	48 (37%)	.145	63 (48%)	.336	97 (74%)	.806	67 (51%)	.263
	≥50	61	30 (49%)		35 (57%)		47 (77%)		38 (62%)	
TNM stage	I	35	13 (37%)	.581	15 (43%)	.625	25 (71%)	.894	20 (57%)	.821
	II	124	49 (40%)		67 (54%)		93 (75%)		65 (52%)	
	III and IV	33	16 (48%)		16 (48%)		26 (79%)		20 (61%)	
T stage	1	55	23 (42%)	.145	26 (47%)	.761	42 (76%)	.957	31 (56%)	1.000
	2	122	45 (37%)		63 (52%)		91 (75%)		66 (54%)	
	3 and 4	15	10 (67%)		9 (60%)		11 (73%)		8 (53%)	
LN metastasis	Absence	102	36 (35%)	.145	51 (50%)	.810	72 (71%)	.304	53 (52%)	.559
	Presence	90	42 (47%)		47 (52%)		72 (80%)		52 (58%)	
Latent distant metastasis	Absence	148	46 (31%)	<.001	64 (43%)	<.001	103 (70%)	.011	69 (47%)	<.001
	Presence	44	32 (73%)		34 (77%)		41 (93%)		36 (82%)	
Histologic type	NST	184	76 (41%)	.382	93 (51%)	.625	137 (74%)	.646	99 (54%)	.381
	Lobular	8	2 (25%)		5 (63%)		7 (88%)		6 (75%)	
Tubule formation	>75%	33	10 (30%)	.256	14 (42%)	.052	21 (64%)	.375	13 (39%)	.162
	11-75%	81	31 (38%)		35 (43%)		61 (75%)		43 (53%)	
	<10%	78	37 (47%)		49 (63%)		62 (79%)		49 (63%)	
Nuclear pleomorphism	1	17	3 (18%)	.073	7 (41%)	.144	9 (53%)	.237	7 (41%)	.246
	2	92	34 (37%)		41 (45%)		71 (77%)		46 (50%)	
	3	83	41 (49%)		50 (60%)		64 (77%)		52 (63%)	
Mitoses/10 HPFs	0-9	112	36 (32%)	.010	47 (42%)	.016	78 (70%)	.028	56 (50%)	.115
	10-19	42	18 (43%)		24 (57%)		30 (71%)		23 (55%)	
	>19	38	24 (63%)		27 (71%)		36 (95%)		26 (68%)	
Histologic grade	1	65	19 (29%)	.013	25 (38%)	.003	43 (66%)	.035	26 (40%)	.044
	2	88	35 (40%)		43 (49%)		65 (74%)		53 (60%)	
	3	39	24 (62%)		30 (77%)		36 (92%)		26 (67%)	
HER2	Negative	128	49 (38%)	.382	60 (47%)	.163	98 (77%)	.698	70 (55%)	1.000
	Positive	64	29 (45%)		38 (59%)		46 (72%)		35 (55%)	
ER	Negative	86	42 (49%)	.074	58 (67%)	<.001	63 (73%)	.806	48 (56%)	.889
	Positive	106	36 (34%)		40 (38%)		81 (76%)		57 (54%)	
PR	Negative	92	43 (47%)	.145	55 (60%)	.046	65 (71%)	.364	47 (51%)	.489
	Positive	100	35 (35%)		43 (43%)		79 (79%)		58 (58%)	
BRCA2	Negative	87	13 (15%)	<.001	36 (41%)	.040	45 (52%)	<.001		
	Positive	105	65 (62%)		62 (59%)		99 (94%)			
BRCA1	Negative	48	4 (8%)	<.001	24 (50%)	.868				
	Positive	144	74 (51%)		74 (51%)					
γH2AX	Negative	94	27 (29%)	.004						
	Positive	98	51 (52%)							
PARP1	Negative	114			26 ± 5	.039[†]				
	Positive	78			58 ± 17					

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

[*] The mean number of γH2AX-positive cells ± standard error.

[†] Two-sided *t* test.

and RFS (P = .011; HR, 2.392; 95% CI, 1.226-4.667). The expression of BRCA2 predicted shorter OS (P < .001; HR, 4.284; 95% CI, 2.158-8.505) and RFS (P < .001; HR, 2.886; 95% CI, 1.692-4.925; 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 = .011; RFS, P = .009), BRCA2 (log-rank, OS, P < .001; RFS, P < .001), PARP1 (log-rank, OS, P < .001; RFS, P < .001), and γH2AX (log-rank, OS, P < .001; RFS, P < .001) was significantly associated with shorter OS and RFS (Figure 2*A*). Older age of the patients and higher tumor stage were associated with shorter OS. Among the 166 BCA patients who received postoperative

Figure 1. The expression and prognostic significance of PARP1, γH2AX, and BRCA1 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 μM) for 0.5 hour and lysed for Western blot analysis of BRCA1, BCCA2, PARP1, and γH2AX expression. The treatment of camptothecin increased the expressions of PARP1, γH2AX, BRCA1, and BRCA2. (B) Immunohistochemical expression of PARP1, γH2AX, BRCA1, and BRCA2 in BCA. Original magnification, ×400. (C) The receiver operating characteristic curve analysis for the determination of cutoff points for the immunohistochemical staining scores of PARP1, γH2AX, BRCA1, and 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 γH2AX-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 BRCA2 immunostaining. Cases with scores equal or greater than 13 for PARP1 expression were considered positive. The expression of γH2AX was considered positive when the number of γH2AX-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 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, γH2AX, and BRCA1.

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

Characteristics	No.	OS		RFS	
		HR (95% CI)	*P*	HR (95% CI)	*P*
Age, years, ≥50 (*vs* <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 (0.737-2.979)	.269	1.182 (0.674-2.074)	.56
3	39/192	3.527 (1.723-7.222)	<.001	2.072 (1.123-3.824)	.02
HER2, positive (*vs* negative)	64/192	1.836 (1.079-3.123)	.025	1.608 (1.006-2.569)	.047
ER, negative (*vs* positive)	86/192	1.813 (1.063-3.091)	.029	1.475 (0.929-2.343)	.099
PR, negative (*vs* positive)	92/192	2.125 (1.233-3.662)	.007	2.066 (1.286-3.320)	.003
BRCA2, positive (*vs* negative)	105/192	4.284 (2.158-8.505)	<.001	2.886 (1.692-4.925)	<.001
BRCA1, positive (*vs* negative)	144/192	2.965 (1.269-6.926)	.012	2.392 (1.226-4.667)	.011
PARP1, positive (*vs* negative)	98/192	5.778 (3.143-10.623)	<.001	3.039 (1.889-4.888)	<.001
γH2AX, positive (*vs* 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

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 γH2AX (log-rank, OS, $P < .001$; RFS, $P < .001$) were significantly associated with both OS and RFS (Figure 2*B*).

Among the 33 triple-negative BCAs ($HER2^-/ER^-/PR^-$), PARP1 expression predicted shorter OS ($P = .017$; HR, 12.256; 95% CI, 1.564-96.035) and RFS ($P = .046$; HR, 3.227; 95% CI, 1.023-10.172). γH2AX 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 2*C*).

Furthermore, we evaluated the prognostic effect of the combined expression of PARP1, γH2AX, BRCA1, and BRCA2. When we focused our analysis on the expressional 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 $\gamma H2AX^-$ and $\gamma H2AX^+$ subgroups (Table 3). γH2AX positivity was associated with shorter OS and RFS in the $BRCA1^+$, $BRCA2^+$, and $PARP1^+$ subgroups (Table 3). Because the expressions of PARP1, γH2AX, BRCA1, and BRCA2 were closely related (Table 1), the combined score for the immunohistochemical expression of BRCA1, BRCA2, PARP1, and γH2AX (CSbbph) was established with the sum of positivity of BRCA1, BRCA2, PARP1, and γH2AX (negative, 0; positive, 1; i.e., $BRCA1^+/BRCA2^+/PARP1^+/\gamma H2AX^+ = 1 + 1 + 1 + 1$ = CSbbph 4). The CSbbph ranged from zero ($BRCA1^-/BRCA2^-/PARP1^-/\gamma H2AX^-$) to four ($BRCA1^+/BRCA2^+/PARP1^+/\gamma H2AX^+$). 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).

The Expression of PARP1, γH2AX, and BRCA2, and BRCA1/BRCA2/PARP1/γH2AX Expression Pattern Is the Independent Unfavorable Prognostic Predictor of BCA Patients

The clinicopathologic factors significantly associated with OS and/or RFS by univariate analysis were included in the multivariate analysis (Table 4). Among the 192 cases of BCA, tumor stage, PARP1 expression, γH2AX positivity, and BRCA2 expression were independent prognostic indicators of both OS and RFS. The expression of PARP1 predicted a 3.648-fold (95% CI, 1.885-7.059; $P < .001$) greater risk of death and a 1.958-fold (95% CI, 1.146-3.347; $P = .014$) greater risk of shorter RFS. γH2AX positivity predicted a 3.564-fold (95% CI, 1.793-7.085; $P < .001$) greater risk of death and a 3.077-fold (95% CI, 1.767-5.357; $P < .001$) greater risk of shorter RFS. The expression of BRCA2 predicted a 2.098-fold (95% CI, 1.004-4.382; $P = .049$) greater risk of death and a 1.868-fold (95% CI, 1.025-3.407; $P = .041$) greater risk of shorter RFS (Table 4). The age of the patient ($P = .032$) was an independent prognostic indicator of OS, and loss of PR expression predicted shorter RFS ($P = .021$). When multivariate analysis was performed with the inclusion of CSbbph instead of the individual expression of PARP1, γH2AX, BRCA1, or BRCA2, CSbbph expression was significantly associated with OS ($P < .001$) and RFS ($P < .001$; Table 4).

Among the subpopulation of patients who received chemotherapy or endocrine therapy, tumor stage, the expression of PARP1 and γH2AX, and CSbbph were independent prognostic predictors of OS and RFS. All *P* values were less than .05.

Discussion

During the treatment of human malignant tumors with genotoxic agents, the expression of PARP1 and γH2AX is observed in tumor cells and is thought to promote the survival of tumor cells by repairing DNA damage [38]. In agreement with these reports, our result demonstrated the expression of PARP1, γH2AX, and BRCA2 to be independent indicators of poor prognosis of BCA, especially in the subpopulation of BCA patients who received adjuvant chemotherapy. In line with our results, it has been reported that the expression of PARP1 or γH2AX in human malignant tumors is associated with tumor progression and poor survival of patients with human

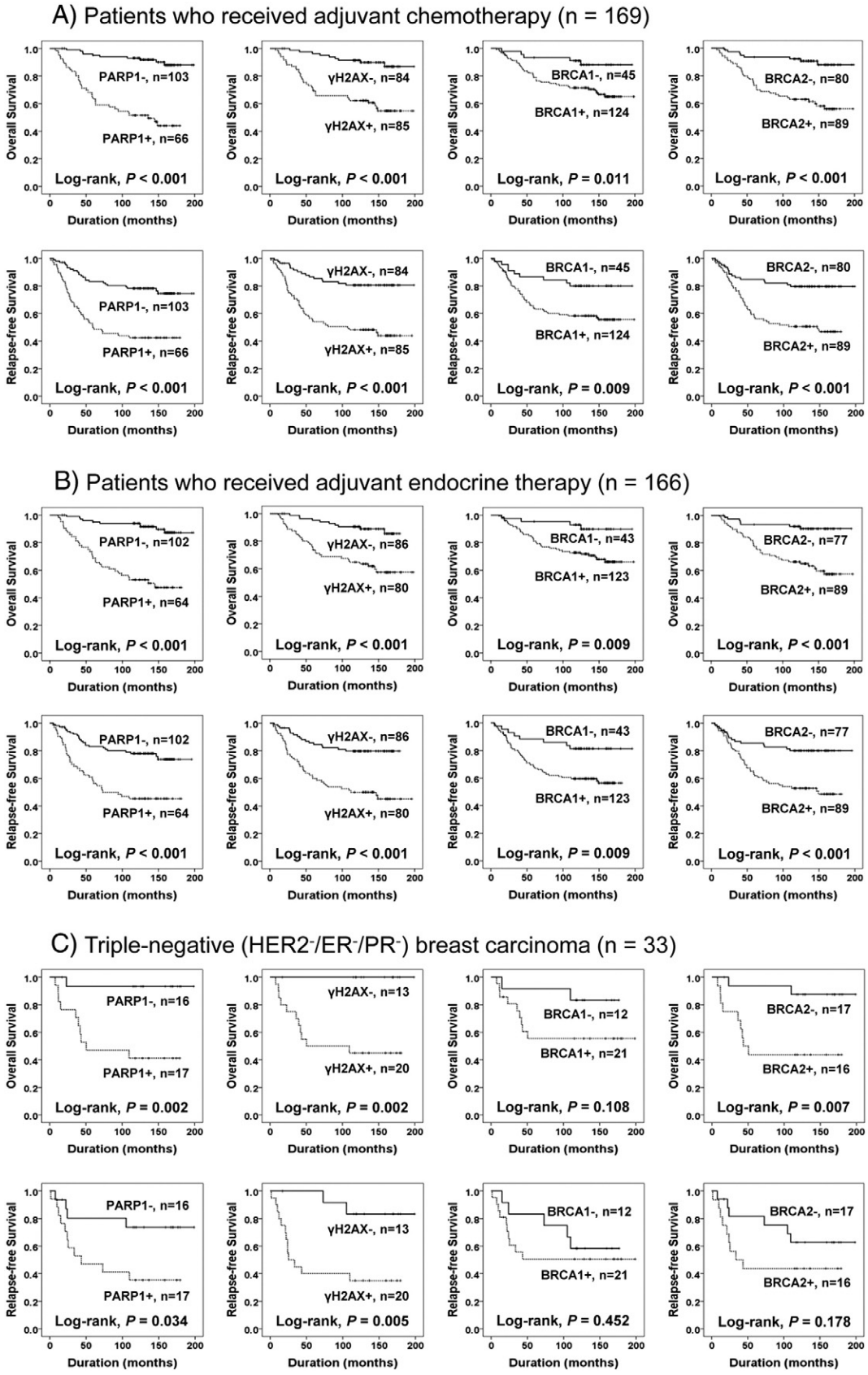


Figure 2. Kaplan-Meier survival analysis according to the expression of PARP1, γH2AX, BRCA1, and BRCA2 in the subpopulations of BCAs. (A) OS and RFS in 169 BCA patients who received adjuvant chemotherapy. (B) OS and RFS in 166 BCA patients who received postoperative endocrine therapy. (C) OS and RFS in 33 triple-negative ($HER2^-/ER^-/PR^-$) BCA patients.

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
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)		
			P	.021	.010	.124	.151	.075	.386		
	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)	2.619 (1.330-5.156)		
			P	<.001	.001	<.001	<.001	.003	.005		
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 (0.509-3.268)
			P	.003	.019	.075	.066			.568	.592
	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)			1.156 (0.280-4.774)	1.615 (0.393-6.633)
			P	<.001	.018	<.001	<.001			.842	.506
γH2AX	Negative	94	HR (95% CI)	3.409 (1.033-11.248)	2.104 (0.830-5.333)			3.155 (0.837-11.894)	1.891 (0.733-4.880)	3.536 (0.452-27.687)	1.931 (0.559-6.671)
			P	.044	.117			.090	.188	.229	.298
	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)	2.967 (1.169-7.531)	2.733 (1.233-6.057)
			P	<.001	<.001			<.001	.002	.022	.013
PARP1	Negative	114	HR (95% CI)			2.632 (0.882-7.858)	2.861 (1.320-6.201)	3.115 (1.042-9.309)	2.432 (1.150-5.146)	1.465 (0.457-4.692)	1.931 (0.820-4.547)
			P			.083	.008	.042	.020	.520	.132
	Positive	78	HR (95% CI)			4.507 (1.891-10.741)	3.577 (1.654-7.733)	1.75 (0.686-4.469)	1.472 (0.622-3.485)	1.101 (0.265-4.562)	0.825 (0.255-2.666)
			P			<.001	.001	.242	.379	.895	.747

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].

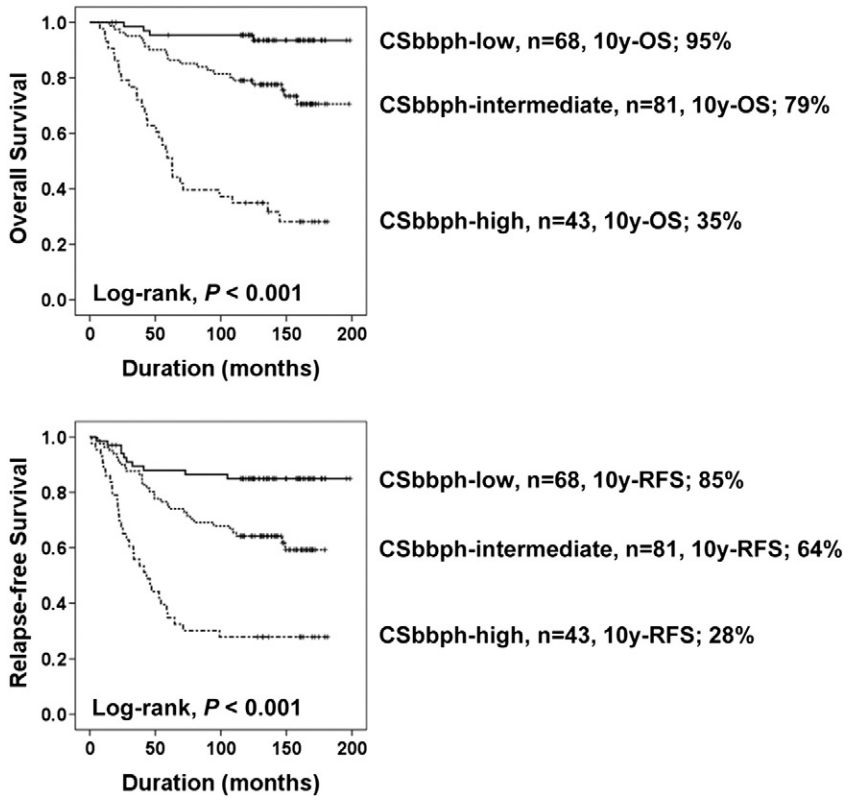


Figure 3. Prognostic significance of the combined expression pattern of PARP1, γH2AX, BRCA1, and BRCA2 in 192 BCAs. Kaplan-Meier survival analysis for OS (A) and RFS (B) between the subgroups classified according to CSbbph. CSbbph was established with the sum of positivity of BRCA1, BRCA2, PARP1, and γH2AX (negative, 0; positive, 1). CSbbph scores were grouped as CSbbph-low (CSbbph 0-1), CSbbph-intermediate (CSbbph 2-3), and CSbbph-high (CSbbph 4); 10y-RFS, RFS rate at 10years.

Table 4. Multivariate Cox Proportional Hazards Regression Analysis for OS and RFS in BCA Patients

Characteristics	OS		RFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Age, years, ≥50 (*vs* <50)	1.830 (1.052-3.182)	.032		
TNM stage				
I	1	.001	1	.011
II	2.641 (0.924-7.544)	.070	2.121 (0.996-4.516)	.051
III and IV	6.676 (2.133-20.899)	.001	3.626 (1.545-8.512)	.003
PR, negative (*vs* positive)	1.733 (0.990-3.032)	.054	1.773 (1.091-2.881)	.021
BRCA2, positive (*vs* negative)	2.098 (1.004-4.382)	.049	1.868 (1.025-3.407)	.041
PARP1, positive (*vs* negative)	3.648 (1.885-7.059)	<.001	1.958 (1.146-3.347)	.014
γH2AX, positive (*vs* negative)	3.564 (1.793-7.085)	<.001	3.077 (1.767-5.357)	<.001
CSbbph †				
Low	1	<.001	1	<.001
Intermediate	3.955 (1.337-11.702)	.013	2.979 (1.457-6.089)	.003
High	17.155 (5.914-49.762)	<.001	7.958 (3.866-16.380)	<.001

* The variables included in the multivariate analysis were age, TNM stage, histologic grade, and the expression of HER2, ER, PR, BRCA1, BRCA2, PARP1, and γH2AX.

† The variables included in the multivariate analysis were age, TNM stage, histologic grade, the expression of HER2, ER, and PR, and CSbbph.

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 G_2/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 expressional 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 the both $BRCA1^-$ and $BRCA1^+$ subgroups. Moreover, the patients with $BRCA1^+/BRCA2^+/PARP1^+/\gamma H2AX^+$ 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 BRCA-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 2*C*, 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2015.04.004.

Acknowledgements

We thank D. B. Leveson-Gower for providing the medical writing services.

References

[1] Carey LA and Sharpless NE (2011). PARP and cancer—if it's broke, don't fix it. *N Engl J Med* **364**, 277–279.

[2] Krishnakumar R and Kraus WL (2010). The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. *Mol Cell* **39**, 8–24.

[3] Redon CE, Nakamura AJ, Zhang YW, Ji JJ, Bonner WM, Kinders RJ, Parchment RE, Doroshow JH, and Pommier Y (2010). Histone γH2AX and poly(ADP-ribose) as clinical pharmacodynamic biomarkers. *Clin Cancer Res* **16**, 4532–4542.

[4] Ossovskaya V, Koo IC, Kaldjian EP, Alvares C, and Sherman BM (2010). Upregulation of poly (ADP-ribose) polymerase-1 (PARP1) in triple-negative breast cancer and other primary human tumor types. *Genes Cancer* **1**, 812–821.

[5] Rojo F, Garcia-Parra J, Zazo S, Tusquets I, Ferrer-Lozano J, Menendez S, Eroles P, Chamizo C, Servitja S, and Ramírez-Merino N (2012). Nuclear PARP-1 protein overexpression is associated with poor overall survival in early breast cancer. *Ann Oncol* **23**, 1156–1164.

[6] Gan A, Green AR, Nolan CC, Martin S, and Deen S (2013). Poly(adenosine diphosphate-ribose) polymerase expression in BRCA-proficient ovarian high-grade serous carcinoma; association with patient survival. *Hum Pathol* **44**, 1638–1647.

[7] Staibano S, Pepe S, Lo Muzio L, Somma P, Mascolo M, Argenziano G, Scalvenzi M, Salvatore G, Fabbrocini G, and Molea G, et al (2005). Poly(adenosine diphosphate-ribose) polymerase 1 expression in malignant melanomas from photoexposed areas of the head and neck region. *Hum Pathol* **36**, 724–731.

[8] Galia A, Calogero AE, Condorelli R, Fraggetta F, La Corte A, Ridolfo F, Bosco P, Castiglione R, and Salemi M (2012). PARP-1 protein expression in glioblastoma multiforme. *Eur J Histochem* **56**, e9.

[9] Barazzuol L, Jena R, Burnet NG, Meira LB, Jeynes JC, Kirkby KJ, and Kirkby NF (2013). Evaluation of poly (ADP-ribose) polymerase inhibitor ABT-888 combined with radiotherapy and temozolomide in glioblastoma. *Radiat Oncol* **8**, 65.

[10] Chow JP, Man WY, Mao M, Chen H, Cheung F, Nicholls J, Tsao SW, Li Lung M, and Poon RY (2013). PARP1 is overexpressed in nasopharyngeal carcinoma and its inhibition enhances radiotherapy. *Mol Cancer Ther* **12**, 2517–2528.

[11] Tentori L, Muzi A, Dorio AS, Bultrini S, Mazzon E, Lacal PM, Shah GM, Zhang J, Navarra P, and Nocentini G, et al (2008). Stable depletion of poly (ADP-ribose) polymerase-1 reduces in vivo melanoma growth and increases chemosensitivity. *Eur J Cancer* **44**, 1302–1314.

[12] García-Parra J, Dalmases A, Morancho B, Arpí O, Menendez S, Sabbaghi M, Zazo S, Chamizo C, Madoz J, and Eroles P (2014). Poly (ADP-ribose) polymerase inhibition enhances trastuzumab antitumour activity in HER2 overexpressing breast cancer. *Eur J Cancer* **50**, 2725–2734.

[13] Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, Friedlander M, Arun B, Loman N, and Schmutzler RK (2010). Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. *Lancet* **376**, 235–244.

[14] Bonner WM, Redon CE, Dickey JS, Nakamura AJ, Sedelnikova OA, Solier S, and Pommier Y (2008). γH2AX and cancer. *Nat Rev Cancer* **8**, 957–967.

[15] Dickey JS, Redon CE, Nakamura AJ, Baird BJ, Sedelnikova OA, and Bonner WM (2009). H2AX: functional roles and potential applications. *Chromosoma* **118**, 683–692.

[16] Redon C, Pilch D, Rogakou E, Sedelnikova O, Newrock K, and Bonner W (2002). Histone H2A variants H2AX and H2AZ. *Curr Opin Genet Dev* **12**, 162–169.

[17] Kinner A, Wu W, Staudt C, and Iliakis G (2008). γ-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. *Nucleic Acids Res* **36**, 5678–5694.

[18] Podhorecka M, Skladanowski A, and Bozko P (2010). H2AX phosphorylation: its role in DNA damage response and cancer therapy. *J Nucleic Acids* , 920161.

[19] Redon CE, Nakamura AJ, Martin OA, Parekh PR, Weyemi US, and Bonner WM (2011). Recent developments in the use of γ-H2AX as a quantitative DNA double-strand break biomarker. *Aging (Albany NY)* **3**, 168–174.

[20] Rothkamm K and Lobrich M (2003). Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A* **100**, 5057–5062.

[21] Nagelkerke A, van Kuijk SJ, Sweep FC, Nagtegaal ID, Hoogerbrugge N, Martens JW, Timmermans MA, van Laarhoven HW, Bussink J, and Span PN (2011). Constitutive expression of γ-H2AX has prognostic relevance in triple negative breast cancer. *Radiother Oncol* **101**, 39–45.

[22] Mhawech-Fauceglia P, Wang D, Kim G, Sharifian M, Chen X, Liu Q, Lin YG, Liu S, and Pejovic T (2014). Expression of DNA repair proteins in endometrial cancer predicts disease outcome. *Gynecol Oncol* **132**, 593–598.

[23] Brunner AH, Hinterholzer S, Riss P, Heinze G, Weiss K, and Brustmann H (2011). Expression of γ-H2AX in endometrial carcinomas: an immunohistochemical study with p53. *Gynecol Oncol* **121**, 206–211.

[24] Ivashkevich A, Redon CE, Nakamura AJ, Martin RF, and Martin OA (2012). Use of the γ-H2AX assay to monitor DNA damage and repair in translational cancer research. *Cancer Lett* **327**, 123–133.

[25] Tutt A, Robson M, Garber J, Domchek S, Audeh M, Weitzel J, Friedlander M, and Carmichael J (2009). Phase II trial of the oral PARP inhibitor olaparib in BRCA-deficient advanced breast cancer. *J Clin Oncol* **27**, CRA501.

[26] Isakoff S, Overmoyer B, Tung N, Gelman R, Giranda V, Bernhard K, Habin K, Ellisen L, Winer E, and Goss P (2010). A phase II trial of the PARP inhibitor veliparib (ABT888) and temozolomide for metastatic breast cancer. *J Clin Oncol* **28**, 1019.

[27] Gelmon KA, Tischkowitz M, Mackay H, Swenerton K, Robidoux A, Tonkin K, Hirte H, Huntsman D, Clemons M, and Gilks B, et al (2011). Olaparib in

patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study. *Lancet Oncol* **12**, 852–861.

[28] Garg K, Levine DA, Olvera N, Dao F, Bisogna M, Secord AA, Berchuck A, Cerami E, Schultz N, and Soslow RA (2013). BRCA1 immunohistochemistry in a molecularly characterized cohort of ovarian high-grade serous carcinomas. *Am J Surg Pathol* **37**, 138–146.

[29] Lakhani SR, Ellis IO, Schnitt SJ, Tan PH, and Van de Vijver MJ (2012). WHO Classification of Tumours of the Breast. Lyon, France: IARC; 2012 .

[30] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, and Trotti A (2010). AJCC Cancer Staging Handbook: From the AJCC Cancer Staging Manual. New York, NY: Springer; 2010 .

[31] Allred D, Harvey JM, Berardo M, and Clark GM (1998). Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol* **11**, 155–168.

[32] Kim JR, Moon YJ, Kwon KS, Bae JS, Wagle S, Yu TK, Kim KM, Park HS, Lee J-H, and Moon WS (2013). Expression of SIRT1 and DBC1 is associated with poor prognosis of soft tissue sarcomas. *PLoS One* **8**, e74738.

[33] Noh SJ, Bae JS, Jamiyandorj U, Park HS, Kwon KS, Jung SH, Youn HJ, Lee H, Park BH, and Chung MJ, et al (2013). Expression of nerve growth factor and heme oxygenase-1 predict poor survival of breast carcinoma patients. *BMC Cancer* **13**, 516.

[34] Kim JR, Moon YJ, Kwon KS, Bae JS, Wagle S, Kim KM, Park HS, Lee H, Moon WS, and Chung MJ, et al (2013). Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas. *PLoS One* **8**, e82870.

[35] Kang MJ, Kim KM, Bae JS, Park HS, Lee H, Chung MJ, Moon WS, Lee DG, and Jang KY (2013). Tumor-infiltrating PD1-positive lymphocytes and FoxP3-positive regulatory T cells predict distant metastatic relapse and survival of clear cell renal cell carcinoma. *Transl Oncol* **6**, 282–289.

[36] Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, Allred DC, Bartlett JM, Bilous M, and Fitzgibbons P, et al (2014). Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. *Arch Pathol Lab Med* **138**, 241–256.

[37] Mylona E, Melissaris S, Nomikos A, Theohari I, Giannopoulou I, Tzelepis K, and Nakopoulou L (2014). Effect of BRCA1 immunohistochemical localizations on prognosis of patients with sporadic breast carcinomas. *Pathol Res Pract* **210**, 533–540.

[38] Bertram C and Hass R (2008). Cellular responses to reactive oxygen species-induced DNA damage and aging. *Biol Chem* **389**, 211–220.

[39] Pyndiah S, Tanida S, Ahmed KM, Cassimere EK, Choe C, and Sakamuro D (2011). c-MYC suppresses BIN1 to release poly(ADP-ribose) polymerase 1: a mechanism by which cancer cells acquire cisplatin resistance. *Sci Signal* **4**, ra19.

[40] Yan J and Tang D (2014). Prostate cancer stem-like cells proliferate slowly and resist etoposide-induced cytotoxicity via enhancing DNA damage response. *Exp Cell Res* **328**, 132–142.

[41] Donizy P, Pietrzyk G, Halon A, Kozyra C, Gansukh T, Lage H, Surowiak P, and Matkowski R (2014). Nuclear-cytoplasmic PARP-1 expression as an unfavorable prognostic marker in lymph node negative early breast cancer: 15-year follow-up. *Oncol Rep* **31**, 1777–1787.

[42] Santarpia L, Iwamoto T, Di Leo A, Hayashi N, Bottai G, Stampfer M, André F, Turner NC, Symmans WF, and Hortobágyi GN (2013). DNA repair gene patterns as prognostic and predictive factors in molecular breast cancer subtypes. *Oncologist* **18**, 1063–1073.

[43] Marcus JN, Watson P, Page DL, Narod SA, Lenoir GM, Tonin P, Linder-Stephenson L, Salerno G, Conway TA, and Lynch HT (1996). Hereditary breast cancer: pathobiology, prognosis, and BRCA1 and BRCA2 gene linkage. *Cancer* **77**, 697–709.

[44] Nilsson MP, Hartman L, Idvall I, Kristoffersson U, Johannsson OT, and Loman N (2014). Long-term prognosis of early-onset breast cancer in a population-based cohort with a known BRCA1/2 mutation status. *Breast Cancer Res Treat* **144**, 133–142.

[45] Verhoog L, Brekelmans C, Seynaeve C, Van den Bosch L, Dahmen G, Van Geel A, Tilanus-Linthorst M, Bartels C, Wagner A, and Van den Ouweland A (1998). Survival and tumour characteristics of breast-cancer patients with germline mutations of BRCA1. *Lancet* **351**, 316–321.

[46] Huzarski T, Byrski T, Gronwald J, Górski B, Domagała P, Cybulski C, Oszurek O, Szwiec M, Gugała K, and Stawicka M (2013). Ten-year survival in patients With BRCA1-negative and BRCA1-positive breast cancer. *J Clin Oncol* **31**, 3191–3196.

[47] Cho D, Park H, Park SH, Kim K, Chung M, Moon W, Kang M, and Jang K (2015). The expression of DBC1/CCAR2 is associated with poor prognosis of ovarian carcinoma. *J Ovarian Res* **8**, 2.

[48] Bolton KL, Chenevix-Trench G, Goh C, Sadetzki S, Ramus SJ, Karlan BY, Lambrechts D, Despierre E, Barrowdale D, and McGuffog L, et al (2012). Association between BRCA1 and BRCA2 mutations and survival in women with invasive epithelial ovarian cancer. *JAMA* **307**, 382–390.

[49] Nicum S, Roberts C, Boyle L, Kopijasz S, Gourley C, Hall M, Montes A, Poole C, Collins L, and Schuh A, et al (2014). A phase II clinical trial of 6-mercaptopurine (6MP) and methotrexate in patients with BRCA defective tumours: a study protocol. *BMC Cancer* **14**, 983.

[50] Wiedemeyer WR, Beach JA, and Karlan BY (2014). Reversing platinum resistance in high-grade serous ovarian carcinoma: targeting BRCA and the homologous recombination system. *Front Oncol* **4**, 34.

[51] Elstrodt F, Hollestelle A, Nagel JH, Gorin M, Wasielewski M, van den Ouweland A, Merajver SD, Ethier SP, and Schutte M (2006). BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res* **66**, 41–45.