Showa Univ J Med Sci **25**(2), 83～91, June 2013

Original

Poly ADP-ribose Polymerase (PARP) Staining for Immunohistological Investigation of Primary Breast Cancer

Genki TSUKUDA[1,2], Yuko DATE[1], Kunio ASONUMA[1], Yusuke WADA[1], Masayuki ISOZAKI[1,2], Tomoko INAGAKI[1], Mutsuko OMATU[3], Toshiaki KUNIMURA[3], Seigo NAKAMURA[4], Miki KUSHIMA[5], Kazuhide KUMAGAI[2] and Toshio MOROHOSHI[1]

Abstract: Given that clinical trials of poly ADP-ribose polymerase (PARP) 1 inhibitors are underway, in the present study we investigated the prevalence of triple-negative breast cancer and PARP1 expression in patients with primary invasive breast cancer. Immunohistological studies plus PARP staining were performed on samples from 206 primary breast cancer patients undergoing surgery at Showa University Hospital between January 2010 and May 2011. Fifteen patients (7.3%) were found to have triple-negative breast cancer. Hormone receptor-positive patients were significantly more likely to be PARP1 negative. There were no PARP1-negative patients in the triple-negative group. However, there was no significant difference in the rate of PARP1 negativity between patients with triple-negative breast cancer and those with other breast cancer subtypes. There were no PARP1-negative patients in the triple-negative breast cancer group. Given that the effectiveness of PARP inhibitors has not been sufficiently established in clinical trials, a more in-depth analysis is required to determine the factors contributing to effective treatment. Future studies should include more subjects with triple-negative breast cancer and those with *BRCA* mutations.

Key words: breast cancer, subtype, poly ADP-ribose polymerase (PARP) 1, immunohistochemistry

Introduction

In the past, the classification of breast cancer was based largely on conventional histopathological diagnosis. Recently, a hierarchical clustering method based on gene expression profiling was shown to be useful for predicting treatment responsiveness and outcomes[1-3]. Using gene expression profiling, breast cancer can be classified into intrinsic subtypes through detailed gene expression analysis. In clinical practice, immunohistochemistry (IHC) can be a useful

[1] *Department of Pathology, Showa University School of Medicine,* 1-5-8, Hatanodai, Shinagawa-Ku, Tokyo, 142-8555, Japan.
[2] *Department of Surgery, Showa University Toyosu Hospital.*
[3] *Department of Pathology Clinico-diagnostic Pathology, Showa University Northern Yokohama Hospital.*
[4] *Department of Surgery, Division of Breast Surgical Oncology, Showa University School of Medicine.*
[5] *Department of Pathology Clinico-diagnostic Pathology, Showa University School of Medicine.*

surrogate for the gene analysis used for tumor classification[4]. Patients are classified into the following groups on the basis of tumor characteristics: (i) luminal A, consisting of patients who are positive for estrogen (ER) and/or progesterone (PR) receptors, but not human epidermal growth factor receptor 2 (HER2), and who have Ki67 levels ≥14%; (ii) luminal B, consisting of patients who are ER and/or PR positive and either HER2 positive and/or with Ki67 levels ≥14%; (iii) HER2, consisting of patients who are HER-2 positive and ER and PR negative; and (iii) triple negative, comprising patients who are negative for all three receptors (ER, PR, and HER2)[5].

Currently, the treatment for breast cancer is largely decided on the basis of hormone receptor (HR) and HER2 status, with tumors classified into subtypes depending on the HR, HER2, and Ki67 status. This classification has an important role in determining both treatment and prognosis. For example, triple-negative breast cancer has a poor prognosis[6] and limited treatment options. Therefore, there is an urgent need to develop new drugs for the treatment of triple-negative breast cancer.

One of the characteristics of triple-negative breast cancer is that many of these tumors have DNA-repair defects in cells harboring *BRCA* mutations[7]. This has led to the development of poly ADP-ribose polymerase (PARP) inhibitors for the treatment of triple-negative breast cancer.

PARP is an enzyme involved in the DNA repair process that was discovered independently by scientists in Japan and France approximately 40 years ago[8, 9]. The main role of PARP is to detect and repair DNA single-strand breaks. Because tumor cells that are *BRCA1* or *BRCA2* deficient exhibit defective homologous combination repair of DNA double-strand breaks, it was expected that inhibition of PARP may destabilize the genome, leading to cell apoptosis (Fig. 1).

In 2005, Farmer *et al*[11] and Bryant *et al*[12] reported that *BRCA1*- or *BRCA2*-defective cells have greater sensitivity to PARP inhibitors than wild-type cells. These studies raised expectations for the clinical application of PARP inhibitors, leading to the start of clinical trials for familial breast cancer and familial ovarian cancer patients with *BRCA* mutations.

Some studies demonstrated significantly greater PARP1 upregulation in triple-negative breast cancer than in normal tissues. This observation was confirmed in a Phase II clinical trial, the results of which were reported at a plenary session of the American Society of Clinical Oncology (ASCO) annual meeting in 2009[13]. Although clinical trials of PARP inhibitors are currently underway, the effectiveness of these agents has not been sufficiently established as yet. The expression of the PARP1 in primary breast cancer has many questions.

The aim of the present study was to investigate the prevalence of breast cancer subtypes in a Japanese cohort to establish their clinicopathological features, as well as PARP expression.

Materials and Methods

The subjects of the present study were 206 patients with primary invasive breast cancer who underwent surgery at Showa University Hospital between January 2010 and May 2011. Patients who were undergoing neoadjuvant therapies were excluded from the study. The clinicopathological variables evaluated were age, tumor size, histological type, nuclear grade

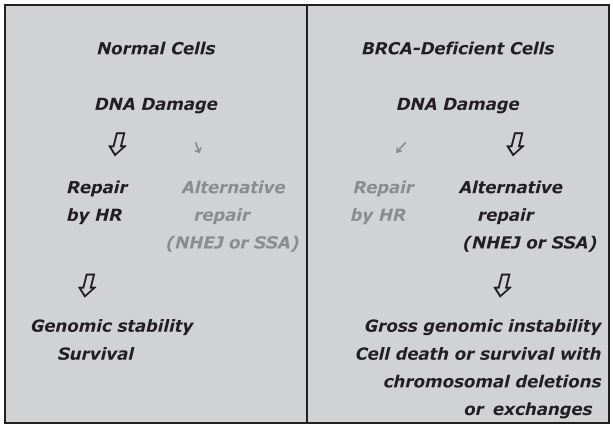

Fig. 1. Loss of functional BRCA1 or BRCA2 affects the choice of the DNA double-strand break (DSB) repair pathway. DNA DSBs are repaired, in part, in normal cells by homologous recombination (HR) -based mechanisms. Functional BRCA1 and BRCA2 proteins are required for efficient repair by HR and genomic stability. In the absence of BRCA1 or BRCA2, alternative repair pathways, such as non-homologous end-joining (NHEJ) and single-strand annealing (SSA), are used, leading to cell death or survival with genomic damage[10].

(NG), ER/PR, HER2, Ki67, lymphovascular invasion, and lymph node metastasis. Tissue specimens were stained immunohistochemically for PARP1 using antibodies obtained from Bethyl Laboratories location and an automated immunohistochemical assay (Ventana HX company). After removal of paraffin, sections were washed and activated by heat treatment with EDTA. Endogenous peroxidase activity was inhibited by hydrogen peroxide solution, followed by blocking of non-specific protein binding. The primary antibodies were diluted 1:250, and the secondary antibodies were conjugated with biotin IgG. Samples were visualized using avidin coupled with horseradish peroxidase, with diaminobenzidine as the chromogen, and enhanced by copper sulfate. Finally, samples were dehydrated, cleared, and mounted after nuclear staining with hematoxylin, as described previously[14, 15].

PAPR1 immunostaining was performed as described by von Minckwits *et al*[14] on sections containing the most invasive areas. Areas showing the closest aggregation of positively stained cells (hot spot) at low magnification were selected for evaluation. Cells showing positive staining were classified on the basis of the intensity of nuclear staining (i.e. percentage of positive cells) as follows: (i) negative staining (0% cells); (ii) weak staining (≥1% to <10% cells); (iii) moderate staining (≥0% to <50% cells); and (iv) strong staining (≥50% cells; Fig. 2).

The ER/PR, HER2, and Ki67 status was evaluated as follows. If ≥10% of cells were positive for ER and PR, the specimen was defined as positive. Being positive for HER2 was defined as IHC results with a score of 3+ (uniform intense membrane staining of ≥30% of

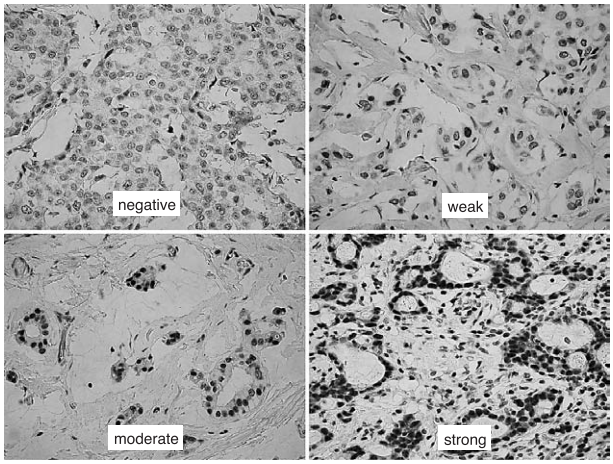

Fig. 2. Evaluation of poly ADP-ribose polymerase (PARP) 1 expression were classified into four groups: (i) negative staining (0% cells); (ii) weak staining (≥1% to <10% cells); (iii) moderate staining (≥0% to <50% cells); and (iv) strong staining (≥50% cells)

invasive tumor cells) according to the 2007 ASCO/College of American Pathologists guideline recommendations for HER2 staining[16]. For Ki67, hot spot abnormalities were evaluated according to the percentage of stained cells (0%～100%, at 10% intervals). The NG was determined as Grade 1, 2, or 3 from the sum of the nuclear atypia and mitotic count scores according to the General Rules for Clinical and Pathological Recording of Breast Cancer[17]. The presence or absence of lymph node metastasis was determined by hematoxylin-eosin staining and immunostaining for cytokeratin. Positive lymph node metastasis was defined as the presence of micro- or macrometastasis (>0.2 mm), whereas the presence of isolated tumor cells (≤0.2 mm) indicated negative lymph node metastasis.

The classification of breast cancer subtypes in the present study was based on the expression patterns of HR (ER/PR), HER2, and Ki67. HR-positive breast cancer was defined as at least ER or PR positive. In the present study, patients were classified into the following four groups: (i) luminal A (HR positive, HER2 negative, Ki67 <20%); (ii) luminal B (HR positive, HER2 positive; or HR positive, HER2 negative, Ki67 ≥20); (iii) HER2 (HR negative, HER2 positive); or (iv) triple negative (HR negative, HER2 negative). We also evaluated the clinicopathological features of the patients in each of these groups.

Statistical analysis

Data among the four groups were compared using Chi-squared analysis for independence (2×4 contingency table). Fisher's exact probability test was used to determine the significance of differences between two groups. Significance was set at $P < 0.05$.

Ethics approval

All patients provided informed consent for all investigations, including the retrieval of personal information from medical records.

Results

The mean age for all 206 patients was 58.1 years (range 27～91 years), with a mean tumor size of 2.03 cm (range 0.04～13.5 cm). Invasive breast cancer was identified histologically in 174 patients (84.5%), with the remaining 32 patients (15.5%) having other types of breast cancer. Vascular invasion was found in 46 patients (22.3%). The NG was estimated to be Grade 1 in 99 patients (56.9%) cases, Grade 2 in 28 patients (16.1%), and Grade 3 in 47 patients (27.0%). Of the 206 patients in the study, 179 (86.9%) were positive for HR and 27 (13.1%) were negative. HER2 was positive in 30 patients (14.6%) and negative in 176 patients (85.4%). Lymph node metastases were present in 60 patients (29.1%) and absent in 164 patients (70.9%). Thirty-six patients were negative for PARP1 36 (17.5%), whereas 45 (21.8%), 53 (25.7%), and 72 (34.9%) patients exhibited weak, moderate, and strong PARP1 staining, respectively (Table 1). Examination of the associations between PARP1 expression and clinicopathological features revealed that significantly more HR-positive patients were PARP1 negative ($P=0.0008$ PARP negative vs. weak staining; $P=0.0006$ PARP negative vs. others). There were no significant differences for any of the other items evaluated (Tables 2, 3).

The prevalence of the luminal A, luminal B, HER2, and triple-negative breast cancer subtypes was 54.4% (n＝112), 32.5% (n＝67), 5.8% (n＝12), and 7.3% (n＝15), respectively. The proportion of patients with each of these subtypes exhibiting negative, weak, moderate, and strong PARP1 expression is given in Table 4. There were no significant differences in the distribution of PARP1 expression among the four groups, although it is of note that there were no PARP1-negative patients in the triple-negative breast cancer group (Table 4).

Discussion

Previous studies have reported that the prevalence of luminal A plus B, HER2, and triple-negative breast cancer subtypes is 60%～80%, 10%～15%, and 10%～20%, respectively[18,19]. In the present study, 86.8% of breast cancers were luminal, 5.8% were HER2 positive, and 7.3% were triple negative, indicating a higher proportion of patients with luminal breast cancer and a lower proportion of patients with triple-negative breast cancer. This is most likely due to the fact that we excluded patients undergoing neoadjuvant therapies from the present study. Generally, patients with HER2-positive or triple-negative breast cancer are more likely to be undergoing neoadjuvant chemotherapies than patients with luminal-type breast cancer.

PARP inhibitors are anticipated to be effective for triple-negative breast cancer because tumors harboring *BRCA* mutations have higher PARP activity[20,21]. However, we did not find any significant differences in PARP expression between patients with triple-negative breast cancer and other breast cancer subtypes in the present study, although there were no PARP1-negative patients in the triple-negative group. PARP1 negativity was significantly more common in the

Table 1. Clinicopathological characteristics of the study subjects

Mean age (years)	58.1	(range: 27 ～ 91)
Mean of tumor size (cm)	2.03	(range: 0.04 ～ 13.5)
Histologic type		
Invasive ductal carcinoma	174	(84.5%)
Special type	32	(15.5%)
Lymphovascular invasion		
negative	160	(77.7%)
positive	46	(22.3%)
Nuclear grade		
1	99	(56.9%)
2	28	(16.1%)
3	47	(27%)
HR		
negative	27	(13.1%)
positive	179	(85.4%)
HER2		
negative (score 0, 1, 2)	176	(85.4%)
positive (score 3)	30	(14.6%)
Lymph node metastasis		
negative	146	(70.9%)
positive	60	(29.1%)
Subtype		
Luminal A	112	(54.4%)
Luminal B	67	(32.5%)
HER2	12	(5.8%)
Triple negative	15	(7.3%)

ER/PR-positive patients. One study as reported the absence of any correlation between PARP activity and PARP expression[22]. The results of the present study suggest that PARP inhibitors may be ineffective for HR-positive breast cancers with *BRCA* mutations. However, a Phase Ⅱ clinical trial of iniparib reported that the clinical benefit rate increased significantly from 34% to 56% ($P = 0.01$) when patients were treated with a combination of gemcitabine and carboplatin[23]. However, the effectiveness of PARP inhibitors has not been sufficiently established in currently ongoing clinical trials[24]. A more in-depth analysis is required to determine the factors contributing to effective treatment. Then we advocate that the effectiveness of these agents be

Table 2. Relationship between the intensity of nuclear staining for PARP1 and clinicopathological characteristics

PARP 1	negative (36)	weak (45)	moderate (53)	strong (72)
Mean age (years)	59.0 (34 ~ 86)	58.6 (29 ~ 91)	56.5 (30 ~ 82)	58.4 (27 ~ 88)
Mean of tumor size (cm)	2.17 (0.05 ~ 6.7)	2.03 (0.2 ~ 9.0)	1.74 (0.2 ~ 5.5)	2.13 (0.04 ~ 13.5)
Histologic type				
Ductal type	33 (91.7%)	37 (82.2%)	42 (79,2%)	62 (86.1%)
Special type	3 (8.3%)	8 (17.8%)	11 (20.8%)	10 (13.9%)
Lymphovascular Invasion				
Negative	28 (77.8%)	37 (81.2%)	42 (79.2%)	56 (77.8%)
Positive	8 (22.2%)	8 (17.8%)	11 (20.8%)	16 (22.2%)
Nuclear grade				
1	23 (63.9%)	22 (48.9%)	30 (56.6%)	46 (63.9%)
2	8 (22.2%)	12 (26.7%)	7 (13.2%)	9 (12.5%)
3	5 (13.9%)	11 (24.4%)	16 (30.9%)	17 (23.6%)
HR				
Negative	1 (2.8%)	12 (26.7%)	7 (13.2%)	8 (11.1%)
Positive	35 (97.2%)	33 (73.3%)	46 (86.8%)	64 (88.9%)
HER2				
Negative	33 (91.7%)	35 (77.8%)	47 (88.7%)	61 (84.7%)
Positive	3 (8.3%)	10 (22.2%)	6 (11.3%)	11 (15.3%)
Lymph node metastasis				
Negative	23 (53.9%)	36 (80.0%)	36 (67.9%)	51 (70.8%)
Positive	13 (36.1%)	9 (20.0%)	17 (32.1%)	21 (29.2%)

Table 3. Relationship between the intensity of nuclear staining for PARP1 and HR status

PARP1	negative (36)	weak (45)	moderate (53)	strong (72)
HR				
Negative	1 (2.8%)	12 (26.7%)	7 (13.2%)	8 (11.1%)
Positive	35 (97.2%)	33 (73.3%)	46 (86.8%)	64 (88.9%)

$^{**}P = 0.0047$

$^{*}P = 0.0342$

$^{*}P < 0.05$, $^{*}P < 0.005$ compared with negative PARP staining.

Table 4. PARP1 expression in different breast cancer subtypes

PARP1	negative	weak	moderate	strong
Luminal A (112 case)	23 (20.5%)	20 (17.9%)	31 (27.7%)	38 (33.9%)
Luminal B (67 case)	12 (17.9%)	13 (19.4%)	15 (22.4%)	27 (40.3%)
HER2 (12 case)	1 (8.3%)	5 (41.7%)	3 (25.0%)	3 (25.0%)
Triple negative (15 case)	0 (0%)	7 (46.7%)	4 (26.7%)	4 (26.7%)

examined in patients.

There are several limitations to the present study. The main weaknesses of the immunohistochemical approaches used herein are their limited technical reproducibility, subjective interpretation, and qualitative readouts. Moreover, although we investigated PARP expression in the present study, we did not evaluate PARP activity.

Future studies into the expression of PARP1 as a biomarker for the therapeutic activity of PARP inhibitor-based therapy should include more subjects with triple-negative breast cancer, as well as those with *BRCA* mutations.

Conflict of interest

The authors have declared no conflict of interest.

References

1) Perou CM, Sorlie T, Eisen MB, *et al*. Molecular portraits of human breast tumours. Nature. 2000;**406**:747-752.
2) Sorlie T, Perou CM, Tibshirani R, *et al*. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;**98**:10869-10874.
3) Sorlie T, Tibshirani R, Parker J, *et al*. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A. 2003;**100**:8418-8423.
4) Cheang MC, Chia SK, Voduc D, *et al*. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;**101**:736-750.
5) Goldhirsch A, Wood WC, Coates AS, *et al*. Strategies for subtypes-dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol. 2011;**22**:1736-1747.
6) Goldhirsch A, Wood WC, Gelber RD, *et al*. Progress and promise: highlights of the international expert consensus on the primary therapy of early breast cancer 2007. Ann Oncol. 2007;**18**:1133-1144.
7) Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. J Clin Oncol. 2008;**26**:2568-2581.
8) Chambon P, Weill JD, Mandel P. Nicotinamide mononucleotide activation of new DNA-dependent polyadenylic acid synthesizing nuclear enzyme. Biochem Biophys Res Commun. 1963;**11**:39-43.
9) Miwa M, Suginuma T. Splitting of the ribose-ribose linkage of poly (adenosine diphosphate-robose) by a calf thymus extract. J Biol Chem. 1971;**246**:6362-6364.
10) Ashworth A. A synthetic lethal therapeutic approach: poly (ADP) ribose polymerase inhibitors for the treatment of cancers deficient in DNA double-strand break repair. J Clin Oncol. 2008;**26**:3785-3790.

11) Farmer H, McCabe N, Lord CJ, *et al*. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature. 2005;**434**:917-921.
12) Bryant HE, Schultz N, Thomas HD, *et al*. Specific killing of BRCA2-deficient tumours with inhibitors of poly (ADP-ribose) polymerase. Nature. 2005;**434**:913-917.
13) O'Shaughnessy J, Osborne C, Pippen J, *et al*. Efficacy of BSI-201, a poly (ADP-ribose) polymerase-1 (PARP1) inhibitor, in combination with gemcitabine/carboplatin (G/C) in patients with metastatic triple-negative breast cancer (TNBC): results of a randomized phase II trial. J Clin Oncol. 2009;**27**(**18S**):3.
14) von Minckwits G, Muller BM, Loibl S, *et al*. Cytoplasmic poly (adenosine diphosphate-ribose) polymerase expression is predictive and prognostic in patients with breast cancer treated with neoadjuvant chemotherapy. J Clin Oncol. 2011;**29**:2150-2157.
15) Hansel DE, Rahman A, House M, *et al*. Met proto-oncogene and insulin-like growth factor binding protein 3 overexpression correlates with metastatic ability in well-differentiated pancreatic endocrine neoplasms. Clin Cancer Res. 2004;**10**:6152-6158.
16) Wolff AC, Hammond ME, Schwartz JN, *et al*. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007;**25**:118-145.
17) The Japanese Breast Cancer Society ed, General rules for clinical and pathological recording of breast cancer. 17th ed. Tokyo: Kanehara Shuppan; 2012. (in Japanese).
18) Brenton JD, Carey LA, Ahmed AA, *et al*. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J Clin Oncol. 2005;**23**:7350-7360.
19) Lerma E, Peiro G, Ramon T, *et al*. Immunohistochemical heterogeneity of breast carcinomas negative for estrogen receptors, progesterone receptors and Her2/neu (basal-like breast carcinomas). Mod Pathol. 2007;**20**:1200-1207.
20) Ossovskaya V, Koo IC, Kaldjian EP, *et al*. Upregulation of poly (ADP-Ribose) polymerase-1 (PARP1) in triple-negative breast cancer and other primary human tumor types. Genes Cancer. 2010;**1**:812-821.
21) Lakhani SR, Van De Vijver MJ, Jacquemier J, *et al*. The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J Clin Oncol. 2002;**20**:2310-2318.
22) Zaremba T, Ketzer P, Cole M, *et al*. Poly (ADP-ribose) polymerase-1 polymorphisms, expression and activity in selected human tumour cell lines. Br J Cancer. 2009;**101**:256-262.
23) O'Shaughnessy J, Osborne C, Pippen JE, *et al*. Iniparib plus chemotherapy in metastatic triple-negative breast cancer. N Engl J Med. 2011;**364**:205-214.
24) O'Shaughnessy J, Schwartzberg LS, Danso MA, *et al*. A randomized phase III study of iniparib (BSI-201) in combination with gemcitabine/carboplatin (G/C) in metastatic triple-negative breast cancer (TNBC). J Clin Oncol. 2011;**29**(**15S**):1007.

[Received December 21, 2012 : Accepted January 22, 2013]