13 Chan, S., Vermi, W., Luo, J., Lucini, L., Rickert, C., Fowler, A. *et al.* STAT1-deficient mice spontaneously develop estrogen receptor alpha-positive luminal mammary carcinomas. *Breast Cancer. Res.* **14,** R16 (2012).
14 Holmes, K. A., Song, J. S., Liu, X. S., Brown, M. & Carroll, J. S. Nkx3-1 and LEF-1 function as transcriptional inhibitors of estrogen receptor activity. *Cancer Res.* **68,** 7380–7385 (2008).
15 Huang, F. -I., Chen, Y. -L., Chang, C. -L., Yuan, R. -H. & Jeng, Y. -M. Hepatocyte growth factor activates Wnt pathway by transcriptional activation of LEF1 to facilitate tumor invasion. *Carcinogenesis* **33,** 1142–1148 (2012).
16 Beauchemin, D., Lacombe, C. & Van Themsche, C. PAX2 is activated by estradiol in breast cancer cells of the luminal subgroup selectively, to confer a low invasive phenotype. *Mol. Cancer* **10,** 148 (2011).
17 Walsh, T., Casadei, S., Coats, K. H., Swisher, E., Stray, S. M., Higgins, J. *et al.* Spectrum of mutations in BRCA1, BRCA2, CHEK2, and TP53 in families at high risk of breast cancer. *JAMA* 295, 1379–1388, 2006).
18 Sehrawat, B., Sridharan, M., Ghosh, S., Robson, P., Cass, C. E., Mackey, J. R. *et al.* Potential novel candidate polymorphisms identified in genome-wide association study for breast cancer susceptibility. *Hum. Genet.* **130,** 529–537 (2011).
19 Higginbotham, K. S. P., Breyer, J. P., Bradley, K. M., Schuyler, P. A., Plummer, Jr W. D., Freudenthal, M. E. *et al.* A multistage association study identifies a breast cancer genetic locus at NCOA7. *Cancer Res.* **71,** 3881–3888 (2011).
20 Prentice, R. L., Huang, Y., Hinds, D. A., Peters, U., Pettinger, M., Cox, D. R. *et al.* Variation in the FGFR2 gene and the effects of postmenopausal hormone therapy on invasive breast cancer. *Cancer Epidem. Biomarker. Prev.* **18,** 3079–3085 (2009).
21 Ghoussaini, M., Fletcher, O., Michailidou, K., Turnbull, C., Schmidt, M. K., Dicks, E. *et al.* Genome-wide association analysis identifies three new breast cancer susceptibility loci. *Nat. Genet.* **44,** 312–318 (2012).
22 Rebbeck, T. R., DeMichele, A., Tran, T. V., Panossian, S., Bunin, G. R., Troxel, A. B. *et al.* Hormone-dependent effects of FGFR2 and MAP3K1 in breast cancer susceptibility in a population-based sample of post-menopausal African-American and European-American women. *Carcinogenesis* **30,** 269–274 (2009).
23 Yoshimoto, N., Nishiyama, T., Toyama, T., Takahashi, S., Shiraki, N., Sugiura, H. *et al.* Genetic and environmental predictors, endogenous hormones and growth factors, and risk of estrogen receptor-positive breast cancer in Japanese women. *Cancer Sci.* **102,** 2065–2072 (2011).
24 Fanale, D., Amodeo, V., Corsini, L. R., Rizzo, S., Bazan, V. & Russo, A. Breast cancer genome-wide association studies: there is strength in numbers. *Oncogene* **31,** 2121–2128 (2012).
25 Turnbull, C., Ahmed, S., Morrison, J., Pernet, D., Renwick, A., Maranian, M. *et al.* Genome-wide association study identifies five new breast cancer susceptibility loci. *Nat. Genet.* **42,** 504–507 (2010).
26 Hamaguchi, M., Nishio, M., Toyama, T., Sugiura, H., Kondo, N., Fujii, Y. *et al.* Possible difference in frequencies of genetic polymorphisms of estrogen receptor α, estrogen metabolism and P53 genes between estrogen receptor-positive and -negative breast cancers. *Jpn. J. Clin. Oncol.* **38,** 734–742 (2008).
27 Perou, C. M., Sørlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A. *et al.* Molecular portraits of human breast tumours. *Nature* **406,** 747–752 (2000).
28 van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M. *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415,** 530–536 (2002).
29 Roh, M. S., Hong, S. H., Jeong, J. S., Kwon, H. C., Kim, M. C., Cho, S. H. *et al.* Gene expression profiling of breast cancers with emphasis of β-catenin regulation. *J. Korean Med. Sci.* **19,** 275–282 (2004).
30 Chan, P., Möller, A., Liu, M. C., Sceneay, J. E., Wong, C. S., Waddell, N. *et al.* The expression of the ubiquitin ligase SIAH2 (seven in absentia homolog 2) is mediated through gene copy number in breast cancer and is associated with a basal-like phenotype and p53 expression. *Breast Cancer Res.* **13,** R19 (2011).
31 Koren, R., Dekel, Y., Sherman, E., Weissman, Y., Dreznik, Z., Klein, B. *et al.* The expression of DCC protein in female breast cancer. *Breast Cancer Res. Treat.* **80,** 215–220 (2003).
32 Zhen-huan, Z., Yamashita, H., Toyama, T., Yamamoto, Y., Kawasoe, T., Ibusuki, M. *et al.* Nuclear corepressor 1 expression predicts response to first-line endocrine therapy for breast cancer patients on relapse. *Chin. Med. J.* **122,** 1764–1768 (2009).
33 Plevova, P., Bouchal, J., Fiurásková, M., Foretová, L., Navrátilová, M., Zapletalová, J. *et al.* PML protein expression in hereditary and sporadic breast cancer. *Neoplasma* **54,** 263–268 (2007).
34 Yang, X., Hao, Y., Ding, Z., Pater, A. & Tang, S.-C. Differential expression of antiapoptotic gene BAG-1 in human breast normal and cancer cell lines and tissues. *Clin. Cancer Res.* **5,** 1816–1822 (1999).
35 Baysal, B. E. A high-resolution integrated map spanning the SDHD gene at 11q23: a 1.1-Mb BAC contig, a partial transcript map and 15 new repeat polymorphisms in a tumour-suppressor region. *Eur. J. Human Genet.* **9,** 121–129 (2001).
36 Ordulu, Z., Dal C., P., Chong, W. W., Choy, K. W., Lee, C., Muto, M. G. *et al.* Disseminated peritoneal leiomyomatosis after laparoscopic supracervical hysterectomy with characteristic molecular cytogenetic findings of uterine leiomyoma. *Genes Chromosom. Cancer* **49,** 1152–1160 (2010).
37 Cha, P. -C., Takahashi, A., Hosono, N., Low, S. K., Kamatani, N., Kubo, M. *et al.* A genome-wide association study identifies three loci associated with susceptibility to uterine fibroids. *Nat. Genet.* **43,** 447–450 (2011).
38 Carroll, J. S., Meyer, C. A., Song, J., Li, W., Geistlinger, T. R., Eeckhoute, J. *et al.* Genome-wide analysis of estrogen receptor binding sites. *Nat. Genet.* **38,** 1289–1297 (2006).
39 Moffa, A. B. & Ethier, S. P. Differential signal transduction of alternatively spliced FGFR2 variants expressed in human mammary epithelial cells. *J. Cell. Physiol.* **210,** 720–731 (2007).
40 Koziczak, M., Holbro, T. & Hynes, N. E. Blocking of FGFR signaling inhibits breast cancer cell proliferation through downregulation of D-type cyclins. *Oncogene* **23,** 3501–3508 (2004).

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ORIGINAL ARTICLE

Biological characteristics of luminal subtypes in pre- and postmenopausal estrogen receptor-positive and HER2-negative breast cancers

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Abstract
Background Estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negetive breast cancers can be divided into luminal A and luminal B subtypes based on Ki67 expression levels. However, the biological differences in ER and progesterone receptor (PR) expression levels between these luminal subtypes are not clear.
Methods We examined immunohistochemical expression levels of ER, PR, and Ki67 in 180 ER-positive/HER2-negative breast cancers while taking menopausal status into account. Breast cancers were divided according to ER and PR levels (H: >50%, L: ≤50%), and luminal A and B were classified by the Ki67 labeling index (A: Ki67 <14%, B: Ki67 ≥14%).
Results When breast cancers were classified based on ER and PR levels, the distribution of pre- and postmenopausals was significantly different for luminal A ($P < 0.0001$), but not for luminal B cancers. As for luminal A, ER-H/PR-L cancers were rare among premenopausals (8%), but frequent among postmenopausals (54%). Correlation between ER and PR levels among luminal A cancers was strong in premenopausals but weak in postmenopausals. Since crosstalk with growth factor signaling is unlikely in luminal A, we speculate that intratumoral estrogen insufficiency contributed to the characteristics of postmenopausal ER-H/PR-L cancers.
Conclusion We speculate that the biological characteristics of luminal A cancers are influenced by the estrogen environment, but its influence on luminal B cancers may be

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limited. We believe these considerations constitute useful information for a better understanding of the biology of ER-positive-HER2-negetive breast cancers.

Keywords Breast cancer · Luminal subtype · Ki67

Introduction

In clinical settings, breast cancer subtypes have been classified according to estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status. This classification is essentially useful for determining indications for endocrine therapies and/or anti-HER2 therapies. Recently, gene expression profiling of breast cancers has resulted in classification into five distinct types, i.e., luminal A, luminal B, HER2-overexpressing, basal-like, and normal-like [1]. This classification of intrinsic subtypes enables us to further classify the ER-positive/HER2-negative group into luminal A and luminal B subtypes. Since it is not always feasible to obtain gene expression data in daily clinical practice, Cheang et al. [2] have developed a simple classification method of luminal subtypes similar to the intrinsic subtypes by immunohistochemical staining to determine the expression levels of Ki67, a marker of cell proliferation. They identified the optimal cutoff point for the Ki67 labeling index as 13.25% for distinguishing luminal B from luminal A with a sensitivity of 72% [95% confidence interval (CI) 59–82%] and specificity of 77% (95% CI 67–85%). On the basis of this finding, the 12th St. Gallen International Breast Cancer Conference (2011) Expert Panel adopted a new immunohistochemical classification of intrinsic subtypes, essentially by dividing ER-positive/HER2-negative cancers into luminal A and luminal B following the application of the Ki67 labeling index using 14% as the cutoff value [3]. Although luminal subtypes divided by Ki67 are essentially useful, there is no doubt that evaluation of ER and PR expression levels would provide important additional information for understanding the characteristics of estrogen-dependent breast cancers.

Estrogen dependency can be determined basically from ER and PR expression levels, and tumors with high values for both ER and PR seem to show higher responsiveness to endocrine therapies [4]. However, it is not clear whether tumors with low ER expression levels are dependent on estrogen or if PR expression levels can be low even though ER levels are high. It is conceivable that PR is downregulated not only as a result of estradiol deficiency, but also of crosstalk with growth factor signaling [5]. Although it has been speculated that there are two mechanisms of PR downregulation in ER-positive tumors and that sensitivity to endocrine therapy may differ as a result, identifying and distinguishing these two mechanisms clinically seems to be difficult. If cancers are involved in crosstalk with growth factor signaling preferentially occurring in the luminal B subset, PR may be downregulated irrespective of the amount of estrogen. On the other hand, it is speculated that among cancers without growth factor signaling, insufficient estrogen environment possibly recognized in postmenopausal status induces PR downregulation.

The purpose of our study was to clarify how the estrogen environment influences ER and PR expression levels in breast cancers while taking both luminal subtypes and menopausal status into consideration.

Patients and methods

Tumor samples

Consecutive breast cancer patients who underwent mastectomy or breast-conserving surgery at the Hyogo College of Medicine during the period from August 1999 to July 2011 were recruited. From among these patients, formalin-fixed and paraffin-embedded tumor samples were obtained from 180 cases with ER-positive (nuclear staining more than 1%) and HER2-negative (score 0, 1, 2, and FISH-negative). All breast cancers were histologically diagnosed as invasive ductal carcinomas ($n = 159$), invasive lobular carcinomas ($n = 7$), or other types ($n = 14$), and patients with non-invasive carcinoma or those who had received chemotherapy or hormonal therapy before surgery were excluded. Nuclear grade was determined according to the Japanese Breast Cancer Society classification [6]. Informed consent was obtained from all patients, and the study protocol was approved by the Ethics Committee of the Hyogo College of Medicine.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut from tumor samples and used for further immunohistochemical staining. Expression levels of ER (1D5; Dako, Glostrup, Denmark), PR (PgR636; Dako), HER2 (Hercep Test; Dako), and Ki67 (MIB1; Dako) were determined immunohistochemically in terms of the percentage of positive cancer cells in the nuclei for ER, PR, and Ki67, and membrane staining for HER2 used automated immunostainers (BOND-MAX, Leica Microsystems, Tokyo, Japan, for ER and PR, and Autostainer, Dako, for HER2 and Ki67).

Different areas of densely stained lesions were selected microscopically, and nearly 1,000 cancer cells were counted. We determined the percentage of positive cancer cells with moderate or intense nuclear staining for ER, PR,

and Ki67. The slides were examined by two observers who were blinded to the clinicopathological features of the patients and re-checked when the evaluations differed. Using a cutoff value of 50%, the tumors were classified into ER-high (H) (>50%) and -low (L) (≤50%), and PR-high (H) (>50%) and -low (L) (≤50%). Luminal A was characterized as Ki67 <14% and luminal B as Ki67 ≥14% according to the criteria defined by Cheang et al. [2].

Statistical analysis

The relationship between luminal subtypes and various clinicopathological characteristics was evaluated using the chi-squared, Mann-Whitney, or Kruskal-Wallis test. Expression levels of ER, PR, and Ki67 were calculated with the Mann-Whitney or Kruskal-Wallis test, or with Pearson's correlation. Differences were considered statistically significant if $P < 0.05$. Ki67 expression levels were compared among subgroups by means of the Mann-Whitney test with Bonferroni correction for multiple comparisons, and significance was set at $P < 0.0083$.

Results

Relationship of luminal subtypes with clinicopathological characteristics

There were 106 luminal A (59%) and 74 luminal B (41%) cancers. As shown in Table 1, there were significantly more tumors with nuclear grade 3 among luminal B (86%) than luminal A (14%) cancers, and PR expression levels of luminal B (24.8%, 5.0–60.0%, median, 25–75 percentile) were significantly lower than those of luminal A (55.2%, 9.4–84.7%). There was no significant difference between luminal subtypes and other clinicopathological factors, that is, menopausal status, tumor size, histological type, lymph node metastasis, and ER expression levels (Table 1).

Correlation of ER and PR expression levels with menopausal status or Ki67 labeling index

Since PR expression levels of luminal A and B are different, we determined the Ki67 labeling index for ER and PR expression levels and taking menopausal status into consideration. The results are shown in Fig. 1. Using the cutoff value described in Patients and Methods, we divided ER and PR expression levels into four subgroups, i.e., ER-H/PR-H, ER-H/PR-L, PR-L/ER-H, and ER-L/PR-L. Among the premenopausal patients, Ki67 expression of the ER-H/PR-L group (23.1%, 14.0–29.5%) was significantly higher ($P = 0.003$) than that of the ER-H/PR-H subgroup (9.5%, 5.2–18.8%). Among the postmenopausals, on the other hand, Ki67 of the ER-L/PR-L subgroup (22.5%, 12.9–47.9%) was significantly higher ($P = 0.002$) than that of the ER-H/PR-H subgroup (10.0%, 5.0-18.1%).

The distribution of cancers classified according to ER and PR expression levels in pre- and postmenopausals is shown in Table 2. As for luminal A, the distribution between pre- and postmenopausals was significantly different ($P = 0.0001$), with nearly 70% of the cancers recognized in the ER-H/PR-H subgroup among the premenopausals, while ER-H/PR-H accounted for 39%, and ER-H/PR-L (54%) was the most frequent type among the postmenopausals. However, luminal B subtype showed no significant difference of ER and PR expression levels between pre- and postmenopausals.

Correlation between ER and PR expression levels in luminal A subtype

In order to exclude the influence of growth factor signaling in which PR may be downregulated through transcriptional suppression in luminal B, we examined correlations between ER and PR expression levels for the luminal A subtype divided into pre- and postmenopausals. The results are shown in Fig. 2. ER and PR expression levels showed significant correlation (correlation coefficient 0.69, $P < 0.0001$) for premenopausals ($n = 36$), but correlation was weak (correlation coefficient 0.26, $P = 0.02$) for postmenopausals ($n = 70$).

Discussion

The most prominent finding of this study is that the biological characteristics of luminal A and luminal B cancers are different. As shown in Table 2, ER and PR expression levels are more significant in luminal A than in luminal B cancers. Our findings indicate that among premenopausals, nearly 70% of luminal A cancers possessed ER-H/PR-H, but that this was rarely detected in ER-H/PR-L. On the other hand, more than 50% of the postmenopausal luminal A cancers were accounted for by the ER-H/PR-L subgroup. PR, a downstream target molecule of estrogen signaling, may be suppressed during activation of growth factor signaling [5]. However, since PR is induced by estrogen signaling, even if tumors express sufficient ER, estradiol deficiency makes it impossible for much PR to be produced, thus resulting in lower expression levels of PR. Since crosstalk with growth factor signaling is unlikely in luminal A cancers, we hypothesize that low PR expression levels indicate an insufficient supply of intratumoral estradiol. Among premenopausals, estrogen deficiency is unlikely because of high levels of circulating estradiole in the blood produced by menstruation. In the postmenopausal estrogen

Table 1 Relationship between luminal subtypes and clinicopathological characteristics

	Luminal A ($n = 106$)[a]	Luminal B ($n = 74$)[a]	P value
Menopausal status			
Pre-	36 (56)[b]	28 (44)	0.59
Post-	70 (60)	46 (40)	
Tumor size			
≤2 cm	71 (65)	39 (35)	0.05
>2 cm	35 (50)	35 (50)	
Histological type			
IDC	90 (57)	69 (43)	0.06
ILC	7 (100)	0 (0)	
Others	9 (64)	5 (36)	
Lymph node metastasis			
No	76 (62)	46 (38)	0.17
Yes	30 (52)	28 (48)	
Nuclear grade			
1	84 (73)	31 (27)	<0.0001
2	18 (49)	19 (51)	
3	4 (14)	24 (86)	
ER expression levels[c]	90.4 (70.5–100)	87.7 (50–100)	0.22
PR expression levels[c]	55.2 (9.4–84.7)	24.8 (5.0–60.0)	0.04

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma

[a] Luminal A: Ki67 <14%, luminal B: Ki67 ≥14%, [b] values in parentheses indicate percentage, [c] % of positive nuclei, median (25th and 75th percentiles)

milieu, however, local production of estrogens may not be sufficient in all cancers and because of relatively lower estrogens cannot induce high levels of PR. Similar with this observation, Yamashita et al. [7] have reported that the frequency of ER-positive and PR-negative cancers in Japanese women over 50 years (14%) was higher than in women aged 50 years or younger (5%).

On the basis of these observations, we speculate the difference between pre- and postmenopausal ER-H/PR-L cancers depends on a mechanism such as the one described above. This hypothesis seems to be partly supported by our observation that ER and PR expression levels strongly correlate in premenopausal luminal A cancers, but only weakly so in postmenopausal luminal A cancers (Fig. 2). As also shown in this figure, a large number of cancers are plotted in the ER-H/PR-L area among postmenopausals. Haynes et al. [8] demonstrated that mRNA expression levels of PR were significantly associated with intratumoral estradiol concentrations in postmenopausal patients. The findings of this study point to the importance of local synthesis of estradiol for PR expression in postmenopausal breast cancers. In order to reach a definitive answer to this issue, direct comparison between intratumoral estradiol levels and PR expression levels is needed. However, we were unable to obtain enough fresh tissues for this assay.

The frequency of ER-L/PR-L in premenopausal luminal A cancers (19%) was higher than that in postmenopausal luminal A cancers (6%). Estrogen independence in ER-positive cancers and the accompanying induction of growth factor signaling have led to the recognition of ER downregulation in both in vitro and in vivo studies [9, 10]. Moreover, under conditions of excessive estrogen, ER downregulation has also been demonstrated in a previous in vitro study [11]. The reason for the high frequency of ER-L/PR-L in premenopausals may be ER downregulation caused by an excessive estrogen supply because crosstalk with growth factor signaling is less likely in luminal A cancers. Although it is not known whether ER is downregulated by high levels of estrogen in all human breast cancer, such downregulation may occur in premenopausal breast cancers. Thus, the difference in the distribution of ER and PR expression levels between pre- and postmenopausals among luminal A cancers may depend on the estrogen environment. As shown in Fig. 1 and Table 2, luminal B cancers are most frequently involved in premenopausal ER-H/PR-L and postmenopausal ER-L/PR-L cancers. Interestingly, the distribution of ER and PR expression levels was similar for pre- and postmenopausal luminal B cancers, and this finding may indicate that growth factor signaling has a stronger influence than estrogen signaling on the expression of ER and PR.

In conclusion, we were able to identify biological differences between luminal A and luminal B cancers. The estrogen environment appears to have a strong effect on ER and PR expression levels of luminal A, but seems to be less important for luminal B cancers. Determination of Ki67 expression levels may thus be useful for evaluation of estrogen dependency, especially of postmenopausal ER-H/PR-L cancers. It is hoped these findings will prove to be useful for a further understanding of the biological

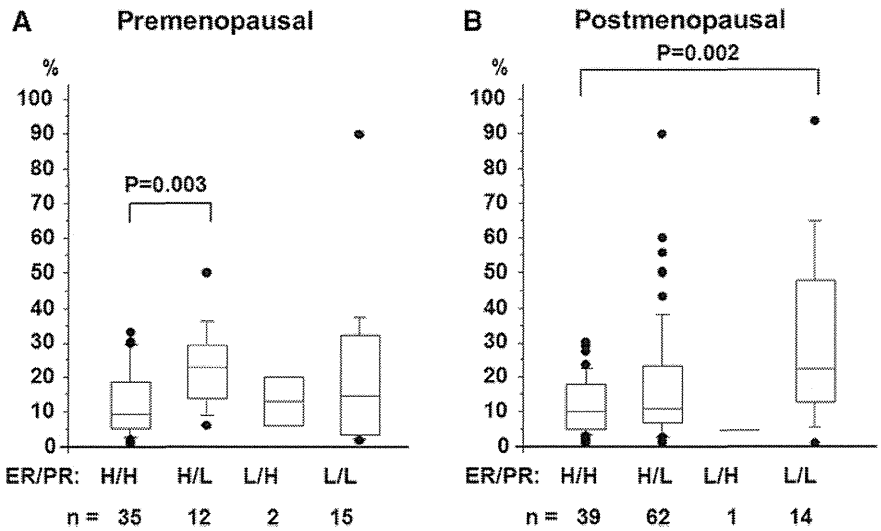

Fig. 1 Ki67 labeling index for ER and PR expression levels in premenopausals (**a**) and postmenopausals (**b**). *H* more than 50% nuclear staining, *L* 50% or less nuclear staining

Table 2 ER, PR expression levels and menopausal status in luminal A and luminal B subtypes

	Premenopausals	Postmenopausals	*P* value
Luminal A[a]			
ER-H and PR-H[b]	25 (69)[c]	27 (39)	0.0001
ER-H and PR-L[b]	3 (8)	38 (54)	
ER-L and PR-H	1 (3)	1 (1)	
ER-L and PR-L	7 (19)	4 (6)	
Luminal B[a]			
ER-H and PR-H	10 (36)	12 (26)	0.25
ER-H and PR-L	9 (32)	24 (52)	
ER-L and PR-H	1 (4)	0 (0)	
ER-L and PR-L	8 (29)	10 (22)	

[a] Luminal A: Ki67 <14%, luminal B: Ki67 ≥14%. [b]H: more than 50% nuclear staining; L: 50% or less nuclear staining, [c] values in parentheses indicate percentage

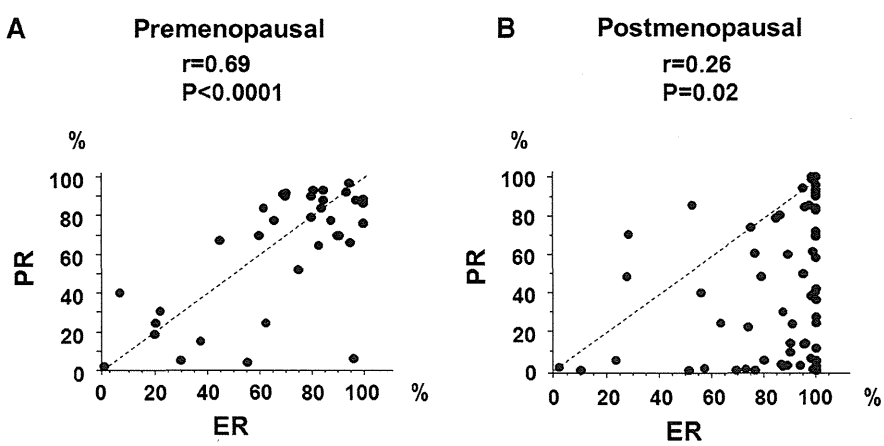

Fig. 2 Correlation between ER and PR expression levels in premenopausal (**a**) and postmenopausal (**b**) luminal A cancers

characteristics of luminal A and luminal B cancers. In order to resolve these issues definitively, studies including larger numbers of patients as well as an investigation of the correlation of the biological characteristics with prognosis need to be done in future.

Conflict of interest Officers or advisers of companies or for-profit organizations: Dr. Toyomasa Katagiri (Memeber of the Board of Oncotherapy Science Co., Ltd). Honoraria paid by companies or for-profit organization as compensation for time or labor of researcher engaged for conference attendance: Dr. Yasuo Miyoshi (Astrazeneca K.K., Taiho Pharmaceutical Co., Ltd, Novartis K.K.).

References

1. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52.
2. Cheang MC, Chia SK, Voduc D, Gao D, Leung S, Snider J, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. J Natl Cancer Inst. 2009;101:736–50.
3. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thürlimann B, Senn HJ. 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;22:1736–47.
4. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet. 2005;365:1687–717.
5. Cui X, Zhang P, Deng W, Oesterreich S, Lu Y, Mills GB, et al. Insulin-like growth factor-I inhibits progesterone receptor expression in breast cancer cells via the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway: progesterone receptor as a potential indicator of growth factor activity in breast cancer. Mol Endocrinol. 2003;17:575–88. Erratum in: Mol Endocrinol. 2003;17:1892
6. Sakamoto G, Inaji H, Akiyama F, Haga S, Hiraoka M, Inai K, et al. Japanese Breast Cancer Society. General rules for clinical and pathological recording of breast cancer 2005. Breast Cancer. 2005;12(Suppl):S1–27.
7. Yamashita H, Iwase H, Toyama T, Takahashi S, Sugiura H, Yoshimoto N, et al. Estrogen receptor-positive breast cancer in Japanese women: trends in incidence, characteristics, and prognosis. Ann Oncol. 2011;22:1318–25.
8. Haynes BP, Straume AH, Geisler J, A'Hern R, Helle H, Smith IE, et al. Intratumoral estrogen disposition in breast cancer. Clinical Cancer Res. 2010;16:1790–801.
9. Brodie A, Sabnis G, Jelovac D. Aromatase and breast cancer. J Steroid Biochem Mol Biol. 2006;102:97–102.
10. Sabnis GJ, Macedo LF, Goloubeva O, Schayowitz A, Brodie AM. Stopping treatment can reverse acquired resistance to letrozole. Cancer Res. 2008;68:4518–24.
11. Harrell JC, Dye WW, Harvell DM, Pinto M, Jedlicka P, Sartorius CA, et al. Estrogen insensitivity in a model of estrogen receptor-positive breast cancer lymph node metastasis. Cancer Res. 2007;67:10582–91.

Genes to Cells

Aberrant DNA methylation status of DNA repair genes in breast cancer treated with neoadjuvant chemotherapy

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Dysregulation of homologous recombination (HR) DNA repair has been implicated in breast carcinogenesis and chemosensitivity. Here, we investigated the methylation status of sixteen HR genes and analyzed their association with tumor subtypes and responses to neoadjuvant chemotherapy. Core specimens were obtained before neoadjuvant chemotherapy from sixty cases of primary breast cancer of the following four subgroups: luminal breast cancer (LBC) with pathological complete response (pCR), LBC with stable disease, triple-negative breast cancer (TNBC) with pCR and TNBC with poor response. The aberrant DNA methylation status of the following HR related-genes was analyzed using bisulfite-pyrosequencing: BRCA1, BRCA2, BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, PALB2, RAD50, RAD51, RAD51C, MRE11, NBS1, CtIP and ATM. Among the genes analyzed, only the incidence of BRCA1 and RNF8 methylation was significantly higher in TNBC than that in LBC. Whereas the incidence of BRCA1 methylation was tended to be higher in pCR cases than in poor-response cases in TNBC, that of RNF8 was significantly lower in pCR cases than in poor-response cases. Our results indicate that the methylation status of HR genes was not generally associated with TNBC subtype or chemosensitivity although hypermethylation of BRCA1 is associated with TNBC subtype and may impact chemosensitivity.

Introduction

Neoplastic transformation of a subset of breast cancers is attributed to dysregulation of DNA repair. In addition to the well-known familial breast cancer genes BRCA1 and BRCA2, hereditary defects in genes required for homologous recombination (HR) DNA repair, such as PALB2/FANCN (Rahman *et al.* 2007), BACH1/BRIP1/FANCJ (Cantor *et al.* 2001; Litman *et al.* 2005), ATM (Renwick *et al.* 2006), NBS1 (Steffen *et al.* 2004), RAD50 (Heikkinen *et al.* 2006) and RAD51C (Meindl *et al.* 2010), have also been implicated in breast cancer susceptibility. The deficiency of HR is important not only for breast cancer susceptibility, but also as a possible target for chemotherapies exploiting synthetic lethality. Inhibitors of poly (ADP-ribose) polymerase and platinum-based agents exhibit significant clinical activity in breast and ovarian cancers caused by BRCA1 or BRCA2 germline mutations in which HR is abrogated (Byrski *et al.* 2009; Audeh *et al.* 2010; Tutt *et al.* 2010). The HR deficiency may also sensitize cells to other DNA damage-inducing agents. For example, basal-like breast cancer, a subtype associated with BRCA1 dysfunction, generally responds to chemotherapy. Sporadic breast cancer with low BRCA1 mRNA expression is reported to be sensitive to anthracycline-based chemotherapy (Margeli *et al.* 2010). *In vitro*, functional deficiency of the genes required

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for the HR pathway sensitizes cells to DNA damage-inducing agents. Thus, it has been suggested that an assessment of HR competence could be valuable in predicting the sensitivity of cancer cells to chemotherapy or specific DNA-damaging agents (Livingston & Silver 2008). However, the contribution of the HR failure caused by genes other than BRCA1 and BRCA2 to the chemosensitivity has not been clarified.

The aberrant DNA methylation status of HR genes in breast cancer tissues has previously been analyzed for BRCA1, BRCA2, PALB2 and ATM. In particular, BRCA1 has been intensively studied (Dobrovic & Simpfendorfer 1997; Magdinier *et al.* 1998; Catteau *et al.* 1999; Esteller *et al.* 2000, 2001; Niwa *et al.* 2000; Rice *et al.* 2000; Alvarez *et al.* 2005; Matros *et al.* 2005; Wei *et al.* 2005, 2008; Turner *et al.* 2007; Chen *et al.* 2009; Xu *et al.* 2009; Branham *et al.* 2012; Cerne *et al.* 2012; Hsu *et al.* 2013; Xu *et al.* 2013). However, in most previous reports, the methylation status was analyzed with methylation-specific restriction enzyme-based assays (Dobrovic & Simpfendorfer 1997; Magdinier *et al.* 1998; Catteau *et al.* 1999; Niwa *et al.* 2000) or methylation-specific PCR (MSP) (Esteller *et al.* 2000, 2001; Rice *et al.* 2000; Alvarez *et al.* 2005; Matros *et al.* 2005; Wei *et al.* 2005, 2008; Turner *et al.* 2007; Chen *et al.* 2009; Xu *et al.* 2009; Hsu *et al.* 2013). The incidence of BRCA1 methylation in breast cancer in these studies varies, and it remains controversial whether aberrant DNA methylation of BRCA1 is preferentially present in basal-like or triple-negative breast cancer (TNBC). DNA methylation analysis can be a useful diagnostic biomarker, but it is important to consider detection methods. Pyrosequencing provides quantitative screening and allows one to set cutoff points, which makes accurate comparisons possible. Recently, BRCA1 methylation in ER α -positive breast cancer analyzed by bisulfite-pyrosequencing was reported (Cerne *et al.* 2012). However, subtype-specificity for the methylation status was not addressed. Moreover, whether tumor response to chemotherapy was associated with the methylation status of HR genes has not been previously addressed.

In this study, we investigated the methylation status of HR genes, including BRCA1 and BRCA2, in primary sporadic breast cancer patients who underwent neoadjuvant anthracycline-based chemotherapy followed by docetaxel by bisulfite-pyrosequencing. We included 15 cases each with pathological complete response (pCR) both in the luminal HER2-negative (LBC) group and in the TNBC group and compared the methylation status of HR genes with that in poor-responding cases. We demonstrate here that the incidence and quantity of methylation in the CpG island of sixteen HR genes in primary breast cancer and investigate their correlation with subtype and chemosensitivity.

Results

Methylation of selected HR markers in breast, colorectal and gastric cancer cell lines

We determined the levels of methylation of all genes in 5 breast cancer cell lines, 8 colorectal cancer cell lines and 11 gastric cancer cell lines and compared them with mixed peripheral blood lymphocytes (PBL) DNA obtained from two healthy male individuals (Table S1 in Supporting Information). Methylation was determined by bisulfite-pyrosequencing, which yields % methylated alleles in the studied DNA (examples in Fig. S1, Supporting Information). The criterion for the assessment of the methylation level of the genes was no methylation, 1–10%; moderate methylation, 11–40%; high methylation, 41–70%; and severe methylation, 71–100%. We found that HERC2, an imprinted gene, is moderately methylated in all the cells, including PBL. BRCC36 is an X-linked gene and is moderately or highly methylated in 6 cell lines. With the exception of the imprinted genes, the incidence of hypermethylation of HR genes was generally low in the cell lines (Table S1 in Supporting Information).

Aberrant DNA methylation status of HR genes in LBC and TNBC

Following sorting due to several limited conditions (Fig. S2 in Supporting Information), we selected 16 HR genes (BRCA1, BRCA2, BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, PALB2, RAD50, RAD51, RAD51C, MRE11, NBS1, CtIP and ATM) to be examined in breast cancer tissue samples. Clinicopathological characteristics of the patients are shown in Table 1. Of the 960 total PCR reactions, 787 (82.0%) reactions were successful (Table S2 in Supporting Information). The other reactions failed despite multiple re-examinations because of insufficient quantity or quality of the DNA samples from the limited amount of core needle biopsy specimens. We first compared the methylation level of the genes in LBC with that

Table 1 Clinicopathological characteristics

	LBC-pCR (n = 15)	LBC-SD (n = 15)	TNBC-pCR (n = 15)	TNBC-PR (n = 15)
Age, median (range)	46.7 (33–63)	51.2 (39–61)	50.1 (27–67)	55.3 (24–76)
Tumor stage				
T1	0	0	6	0
T2	11	10	5	11
T3	4	3	1	2
T4	0	2	3	2
Nodal involvement				
Negative	6	4	2	5
Positive	9	11	13	10
Distant metastasis				
Negative	15	15	15	15
Positive	0	0	0	0
Histological type				
Invasive ductal	15	15	14	13
Metaplastic	0	0	1	1
Apocrine	0	0	0	1
Estrogen receptor status				
Negative	0	0	15	15
Positive	15	15	0	0
Progesteron receptor status				
Negative	4	0	15	15
Positive	11	15	0	0
HER2 status				
Negative	15	15	15	15
Positive	0	0	0	0
Neoadjuvant Chemotherapy				
FEC	3	0	8	3
EC	12	15	7	10
DOC	15	15	15	12
Others	0	0	0	2*
Tumor response				
PD	0	0	0	1
SD	0	15	0	3
PR	0	0	0	11
pCR	15	0	15	0

*One case with six 21-day cycles of TC (docetaxel 75 mg/m^2 and cyclophosphamide 600 mg/m^2) and one case with six 28-day cycles of CMF (cyclophosphamide 200 mg/m^2, methotrexate 40 mg/m^2 and 5-FU 500 mg/m^2 on day 1 and 8).

in TNBC (Fig. 1). Of the 16 genes tested, hypermethylation of BRCA1 and RNF8 was statistically associated with TNBC. Whereas BRCA1 was hypermethylated (14–94%) in 7 of 30 cases of TNBC with a mean methylation level of 14.8 ± 25.4%, it was only moderately methylated (11%) in one of 29 cases of LBC with a mean methylation level of 4.3 ± 1.9% (Fig. 1 and Table S2 in Supporting Information). Although there no cases with high or severe methylation of RNF8 were observed, the incidence of moderate methylation of RNF8 was significantly higher (4/21 cases, 19.0%) in TNBC than that in LBC (0/24 cases, 0%). The mean methylation levels for RNF8 in TNBC and LBC were 6.4 ± 7.1% and 3.0 ± 2.7%, respectively.

The mean methylation level of the majority of HR genes (BRCA2, MDC1, RNF168, PALB2, RAD50, RAD51C, MRE11, CtIP and ATM) tended to be higher in TNBC than in LBC (Fig. 1 and Table S2 in Supporting Information), although there were no significant differences. We also examined the Ki-67 index as an indicator of cell proliferation. The Ki-67

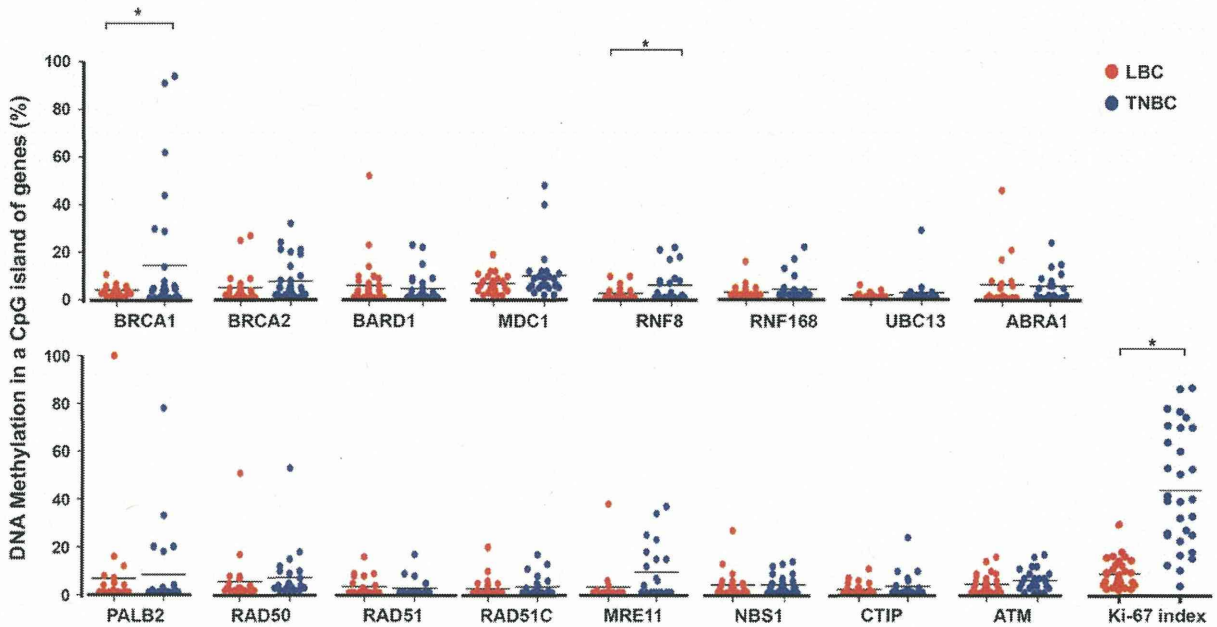

Figure 1 Aberrant DNA methylation status of homologous recombination (HR) genes in luminal breast cancer (LBC) and triple-negative breast cancer (TNBC). Red circles and blue circles indicate percent methylation of HR genes in each case of LBC and TNBC, respectively. The Ki-67 index, as determined by immunohistochemical analysis, is also indicated. Mean percentages are shown as horizontal bars. (*), statistical significance according to Student's *t*-test, BRCA1: $P = 0.03$, RNF8: $P = 0.04$, Ki-67: $P < 0.0001$.

index was significantly higher in the TNBC cases than in LBC (43.6 ± 24.6% vs. 9.0 ± 6.1%, $P < 0.001$) (Fig. 1 and Table S2 in Supporting Information).

Aberrant DNA methylation status of HR genes in pCR cases and poor-responder cases

We next analyzed the association of the methylation level with tumor response to neoadjuvant chemotherapy in TNBC and LBC (Fig. 2). BRCA1 methylation levels tended to be higher in pCR cases than in poor-responder patients in TNBC (Fig. 2B). This tendency was not observed in LBC (Fig. 2A). One metaplastic breast cancer case with severe BRCA1 methylation (94%) in TNBC achieved pCR (Table S2 in Supporting Information). PALB2 methylation levels also tended to be higher in pCR cases in TNBC (Fig. 2B) but not in LBC, with the exception of one case with 100% methylation (Fig. 2A). There were no genes that exhibited higher methylation levels in pCR cases than in SD or poor-response cases with statistical significance. Instead, RNF8 and ATM exhibited lower methylation levels in pCR cases than in poor-response cases (RNF8: 2.7 ± 2.8% vs. 13.7 ± 7.7%, ATM: 5.3 ± 5.0% vs. 8.8 ± 3.8%) in TNBC with statistical significance (RNF8: $P < 0.001$ and ATM: $P = 0.04$, respectively) (Fig. 2B, Table S2 in Supporting Information). The Ki-67 index tended to be higher in SD cases than in pCR cases in both LBC (10.4 ± 6.7% vs. 7.7 ± 5.3%, $P = 0.870$) and TNBC (50.6 ± 23.4% vs. 36.6 ± 24.5%, $P = 0.836$).

Combined hypermethylation of BRCA1, BRCA2, MDC1, ABRA1 and PALB2 with pCR in TNBC

Finally, we examined the individual genes and gene combinations for their association with tumor response by ROC curve analysis. No individual genes exhibited notable AUC values in LBC, in which BRCA2 exhibited the best sensitivity (53.8%), specificity (37.3%) and AUC (0.428) (Fig. 3A). On the other hand, BRCA1 exhibited a relatively high AUC value in TNBC with respect to sensitivity (66.7%), specificity (53.7%) and AUC (0.649) (Fig. 3A). We next analyzed the combinations of the genes in methylation panels that could increase the performance of these markers. Whereas a combination of all 16 genes exhibited a low AUC value (sensitivity: 60.0%, specificity: 40.0%, AUC: 0.489 in LBC, sensitivity:

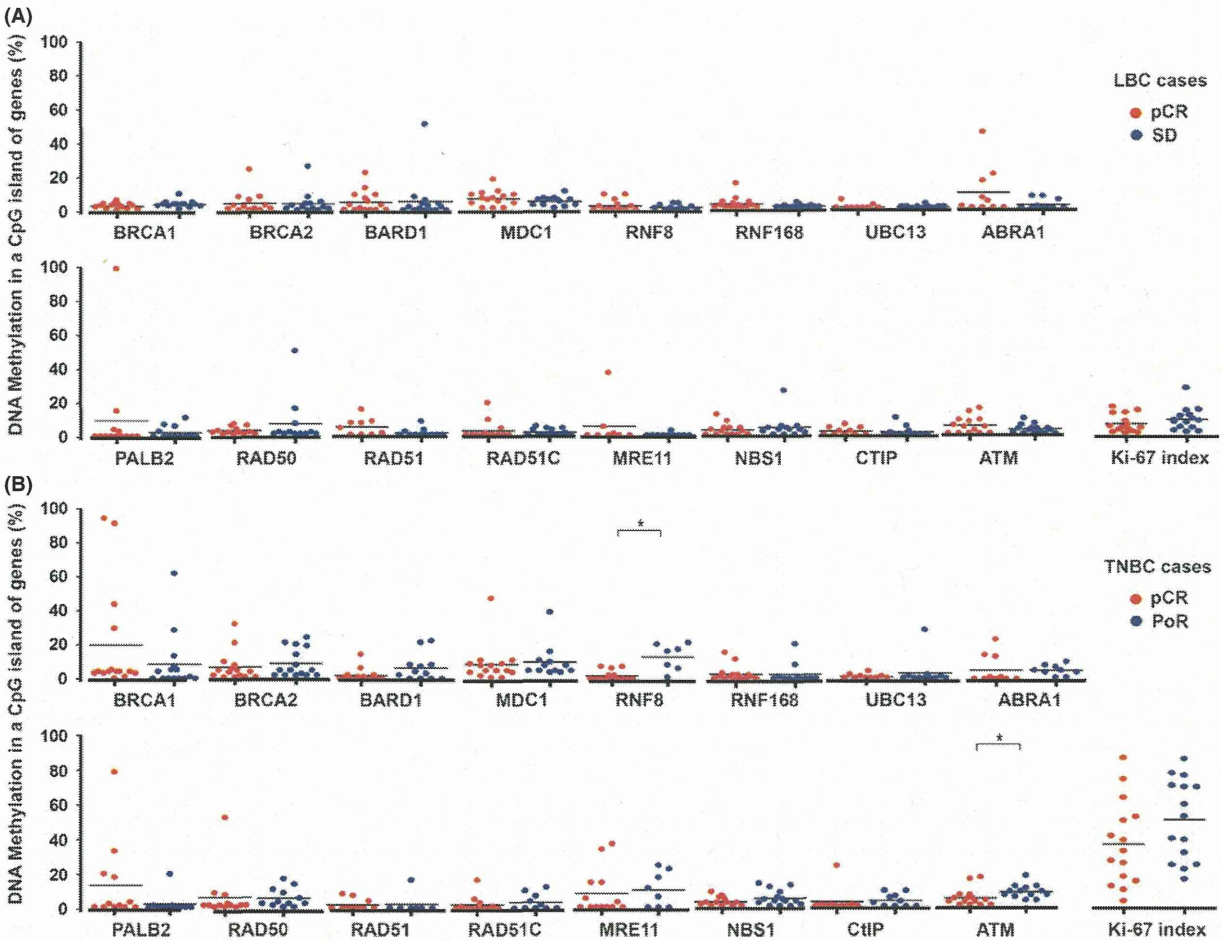

Figure 2 Aberrant DNA methylation status of homologous recombination (HR) genes in cases with pCR and poor response. Red circles and blue circles indicate percent methylation of HR genes in each case of pCR and SD in luminal breast cancer (LBC) (a) or in each case of pCR and poor responder (PoR) in triple-negative breast cancer (TNBC) (b), respectively. The Ki-67 index, as determined by immunohistochemical analysis, is also indicated. Mean percentages are shown as horizontal bars. (*), statistical significance according to Student *t*-test, RNF8: $P < 0.001$, ATM: $P = 0.004$.

73.3%, specificity: 53.3%, AUC: 0.588 in TNBC) (Fig. 3B), a 5-gene combination, BRCA1 + BRCA2 + MDC1 + ABRA1 + PALB2 demonstrated a substantially higher AUC value in TNBC (sensitivity: 73.3%, specificity: 55.6%, AUC: 0.655 in TNBC) (Fig. 3C). The AUC value of the 5-gene combination was rather low in LBC (sensitivity: 60.0%, specificity: 53.3%, AUC: 0.469) (Fig. 3C).

Discussion

In the current study, we investigated the aberrant DNA methylation status of sixteen HR genes in primary sporadic breast cancer cases for their correlation with tumor subtypes and responses to neoadjuvant chemotherapy. Whereas we could detect methylation of only a few of the HR genes in cultured cancer cell lines, we observed substantial methylation in some HR genes in the cancer tissues. There could be some limitation of our study using core needle biopsy specimens as research materials, for example, limited amount of the samples and molecular heterogeneity among different areas within each tumor. However, the core specimen represents tumor characteristics including chemosensitivity in majority of cases in clinical practice.

The correlation between the methylation status of BRCA1 and breast cancer subtype has long been investigated but remains controversial. Using methylation-specific restriction enzyme-based techniques, BRCA1

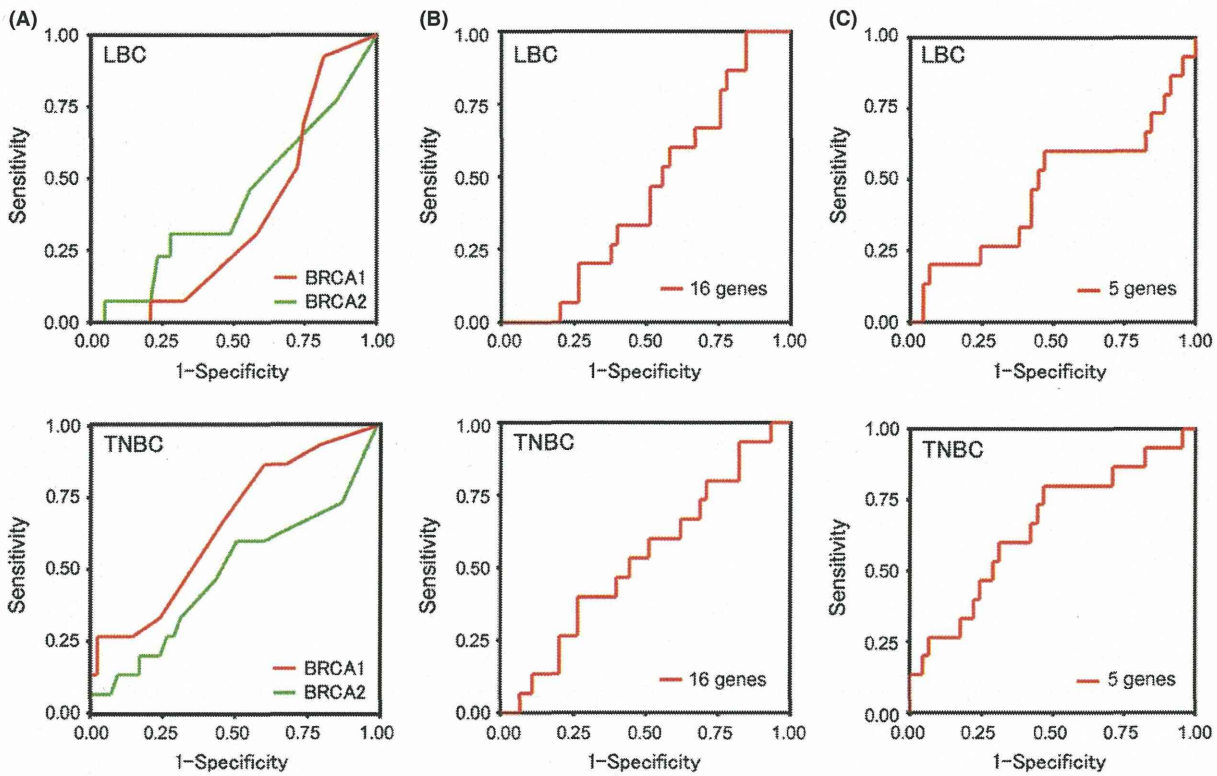

Figure 3 Receiver operating characteristic (ROC) curve of multiple gene panels in luminal breast cancer (LBC) and triple-negative breast cancer (TNBC) samples. The methylation status of BRCA1 and BRCA2 (a), 16 HR gene combination (b) and 5 HR gene (BRCA1 + BRCA2 + MDC1 + ABRA1 + PALB2) combination (c) in their association with tumor response to neoadjuvant chemotherapy in LBC (upper panels) and TNBC (lower panels) samples is shown. *Z*-score s are indicated as red or green polygonal lines.

methylation was reported to be inversely correlated with ERα status (Catteau *et al.* 1999; Niwa *et al.* 2000). Matros *et al.* (Matros *et al.* 2005) investigated the methylation status using MSP and reported that methylation was significantly associated with ERα -positive tumors and was absent in the basal-like subtypes, contradicting the previous reports. However, later studies with MSP were not consistent with the report and demonstrated a relatively higher incidence of BRCA1 methylation in ERα -negative or triple-negative breast cancers (Wei *et al.* 2005, 2008; Chen *et al.* 2009; Hsu *et al.* 2013; Xu *et al.* 2013). The incidence was reported to be remarkably high in special histological types of breast cancer, including medullary, mucinous (Esteller *et al.* 2000) or metaplastic (Turner *et al.* 2007) in TNBC. On the other hand, a recent large cohort of ERα -positive breast cancer examined with pyrosequencing indicated that the BRCA1 methylation level was low with a median value of 2.53% (Cerne *et al.* 2012). In the current study, we also analyzed the methylation status by pyrosequencing for both LBC and TNBC. The results suggest a higher incidence of BRCA1 methylation in TNBC compared with LBC, consistent with the general assumption that BRCA1 deficiency is associated with TNBC and basal-like breast cancer, as is the case with BRCA1 germline mutations or micro-RNA inhibition of BRCA1 (Sorlie *et al.* 2003; Moskwa *et al.* 2011). Furthermore, it is noteworthy that the TNBC-pCR group included 4 cases of highly methylated BRCA1 (30, 44, 91 and 94%) of 15 cases. Interestingly, one case was metaplastic breast cancer, which is generally chemoresistant. It should be noted that the TNBC-pCR group was biased with respect to tumor size; the group contained six T1 cases, whereas the other groups contained no T1 cases (Table 1). This bias is most likely attributable to the fact that small tumors favorably achieve pCR and that TNBC was preferentially treated with neoadjuvant chemotherapy. Interestingly, the T1 cases in TNBC-pCR

included fewer cases with aberrant DNA methylation in HR genes. In contrast, six cases of the remaining 9 cases (66.7%) of TNBC-pCR exhibited remarkable hypermethylation in HR genes, including three BRCA1 cases (44, 91, and 94%), one PALB2 case (78%), one MDC1 case (48%) and one case with moderate methylation of multiple HR genes (MDC1:12%, PALB2:33%, MRE11:15% and ATM:17%) (Table S2 in Supporting Information). Therefore, the contribution of the hypermethylation of HR genes to pCR establishment in TNBC could be underestimated in the current study and may be of greater impact.

For the aberrant DNA methylation status of BRCA2 in breast cancer, the absence of methylation in 18 breast and ovarian cancer cell lines was first reported (Collins *et al.* 1997). This is consistent with our observation that there was no detectable BRCA2 methylation and only a few other methylated HR genes in all of the 24 cancer cell lines analyzed. We speculate that this is presumably due to negative selection of the cells with HR deficiency during cell line establishment. For clinical breast cancer samples, the incidence was reported to range from 15 to 64% using MSP or methyl-specific multiplex-ligation probe amplification assay (MS-MLPA) (Cucer *et al.* 2008; Moelans *et al.* 2011; Branham *et al.* 2012). In our study, 9 of 57 cases (15.8%) exhibited more than 10% methylation in the region analyzed, an incidence consistent with the data reported by Branham *et al.* using MS-MLPA(Branham *et al.* 2012). Seven of the 9 cases were TNBC, although there was no statistically significant difference between LBC and TNBC.

Because the phenotype of BRCA2 germ-line mutation exhibits among all of the subtypes, including LBC and TNBC (Sorlie *et al.* 2003; Jönsson *et al.* 2010), we expected that the aberrant DNA methylation of BRCA2 would also be observed in LBC and that LBC-pCR may exhibit higher incidence of BRCA2 methylation. However, the incidence of methylation was rather low. Interestingly, one case with an extremely high level (100%) of methylation was observed for PALB2 in LBC-pCR group. In this case, PALB2 dysfunction may contribute to the hypersensitivity of the cancer cells to chemotherapy because PALB2 is required for BRCA2 recruitment to damaged DNA sites (Zhang *et al.* 2009). BRCA2 and PALB2 are Fanconi anemia genes FANCD1 and FANCN, respectively, and biallelic loss of these genes causes Fanconi anemia, a genome instability syndrome (Rahman *et al.* 2007), suggesting an indispensable role of PALB2 in DNA damage response.

Germline mutations of a number of HR genes other than BRCA1 and BRCA2 are also implicated in breast cancer susceptibility, indicating that the defect of HR function is associated with breast cancer development. These genes include PALB2, ATM, NBS1, RAD50 and RAD51C. Among them, the aberrant DNA methylation status of PALB2 and ATM in breast cancer tissue has previously been investigated using MSP (Vo *et al.* 2004; Treilleux *et al.* 2007; Potapova *et al.* 2008; Moelans *et al.* 2011). Aberrant DNA methylation of PALB2 was detected in 6 of 68 (8.8%) cases of inherited and sporadic primary breast cancers, including 2 cases of ERα -positive and 4 cases of ERα -negative cancer (Potapova *et al.* 2008). In our study, 8 of 48 (16.7%) cases exhibited more than 10% methylation in the CpG island region analyzed. The discrepancy could be ascribed not only to the different technique used to analyze the methylation, but also to the bias that the current study includes approximately half pCR cases, which exhibit an increased incidence of PALB2 hypermethylation (6/25 cases: 24%). For ATM, the absence (Treilleux *et al.* 2007) or high incidence (18/23 cases: 78%) (Vo *et al.* 2004) of aberrant DNA methylation detected by MSP using the same primers has been reported. Another study with MS-MLPA demonstrated a 12% incidence of hypermethylation in invasive breast cancer (Moelans *et al.* 2011). Our results demonstrated that although the level of methylation was modest (18% at highest), 8 out of 57 cases (14.0%) exhibited methylation in more than 10% of the CpG island region examined.

The aberrant DNA methylation status in breast cancer tissues of other genes, including BARD1, MDC1, RNF8, RNF168, UBC13, ABRA1, RAD 50, RAD51, RAD51C, MRE11, NBS1 and CtIP, has not been previously investigated. Our data indicate that there are some incidences of methylation for these genes. Among them, the incidence of methylation of RNF8 was significantly higher in TNBC than that in LBC. RNF8, an E3 ubiquitin ligase, is one of the upstream proteins in both HR and nonhomologous end-joining pathways and is required for retention of BRCA1-ABRA1-RAP80 complex and 53BP1 at sites of DNA damage (Ohta *et al.* 2011). Down regulation of the two major pathways to repair DNA double-strand breaks caused by RNF8 methylation could be involved in cancer development of TNBC. Although the level of methylation was relatively low, the highest incidence (11/45 cases: 24%)

was observed for MDC1. The incidence of ABRA1 methylation was relatively high in pCR cases (6/20 cases: 30%) than that in poor responders (1/19 cases: 5.3%), although there was no statistical significance. When examining all 16 genes together, we could not detect any association between aberrant DNA methylation status and tumor response to the chemotherapy in either LBC or TNBC. We expected higher incidence of methylation in pCR cases than in poor responders. However, interestingly, significantly higher incidences of methylation were observed in poor responders than in pCR in TNBC for RNF8 and ATM. The biological background underlying this phenomenon is currently unclear. Overall, the levels of methylation of the new HR genes investigated in the current study were not as significant as that of BRCA1, reaffirming the importance of BRCA1 in breast cancer pathogenesis. Nonetheless, we found that a combination of 5 genes, BRCA1, BRCA2, MDC1, ABRA1 and PALB2, possessed the greatest sensitivity, specificity and AUC associated with pCR in TNBC. Hypermethylation of these genes could be a candidate biomarker of breast cancer chemosensitivity and warrants validation in separate data sets.

HER2-negative LBC has been classified as Luminal A and Luminal B subtypes according to a low index and high Ki-67 index, respectively. In general, Luminal B breast cancer is more sensitive to chemotherapy than Luminal A. Therefore, we analyzed the index to clarify whether it associates with pCR. However, pCR cases exhibited even lower levels of Ki-67 when compared with SD or poor-responder cases in both LBC and TNBC, suggesting that a high Ki-67 index is not an efficient marker for chemosensitivity.

In conclusion, this is the first study to quantitatively analyze the aberrant DNA methylation status of HR genes, including BRCA1 and BRCA2, in breast cancer tissues for their association with tumor subtypes and tumor response to chemotherapy. The aberrant DNA methylation status of most of the HR genes studied was not significantly associated with TNBC subtype or chemosensitivity. However, hypermethylation of BRCA1 and RNF8 is associated with TNBC subtype and may impact chemosensitivity. Contrary to our expectation, no severe methylation was observed in LBC with pCR, with the exception of one case that exhibited 100% methylation of PALB2. Because the current study includes only a part of the essential HR genes, future work may be required to identify the possibly missing key HR gene(s) critical for the chemosensitivity.

Experimental procedures

Patients and biopsy specimens

Of the 384 patients with primary invasive breast cancer who underwent neoadjuvant chemotherapy at the Division of Breast and Endocrine Surgery, St. Marianna University School of Medicine, Japan, from August 2007 to August 2010, fifteen sequential cases each of either LBC with pCR, LBC with nonresponding stable disease (SD), TNBC with pCR, or TNBC with poor response were selected for examination. Because the TNBC cases included only 1 case of progressive disease (PD) and 3 cases of SD, an additional 11 cases with partial responses that exhibited the smallest rate of regression in the partial-response cases were analyzed to adjust the total number in the group to 15 cases. HER2-positive patients were excluded because they had been treated with neoadjuvant chemotherapy combined with trastuzumab. Tumor specimens were obtained by core needle biopsy prior to starting therapy for the purpose of diagnosis, and formalin-fixed, paraffin-embedded (FFPE) specimens were stored. Excess specimen samples were analyzed in accordance with an approved Institutional Review Board application of the St. Marianna University School of Medicine (registration number 1865).

Neoadjuvant chemotherapy

The chemotherapy regimen consisted of four 21-day cycles of FEC (5-Fu: 500 mg/m^2, epirubicin: 100 mg/m^2, cyclophosphamide: 500 mg/m^2 on day 1) or EC (epirubicin: 80–90 mg/m^2, cyclophosphamide: 600 mg/m^2 on day 1) followed by four 21-day cycles of DOC (docetaxel: 75 mg/m^2 on day 1), except for two patients in the TNBC-poor-responder group as described in Table 1.

Response evaluation

The pathological response of the tumor to the neoadjuvant chemotherapy was evaluated with hematoxylin-eosin-stained specimens obtained during the primary surgeries. The absence of invasive cancer in the primary lesion following the chemotherapy was defined as pCR. The clinical response was evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.

Gene and primer selection

The HR genes implicated in breast cancer susceptibility were included in the study. In addition, genes evidently involved in and critically functioning in HR (Ciccia & Elledge 2010) that contain appropriate CpG islands around their promoter region were selected as candidates. A flow chart of the process for gene selection is shown in Fig. S2. The sequence and the position of each pair of primers are shown in Table S3 and Fig. S3.

DNA extraction

FFPE specimens were cut in 10 μm sections and subjected to laser-capture microdissection to isolate cancer cells using the PALM Microbeam (Carl Zeiss, Oberkohen, Germany). DNA was extracted using the standard phenol–chloroform method from breast, colorectal and gastric cancer cell lines and microdissected breast cancer tissues.

Bisulfite-pyrosequencing

Bisulfite treatment of gDNA, subsequent polymerase chain reaction (PCR) and pyrosequencing have been performed as described previously (Watanabe *et al.* 2009). All of the primers and PCR conditions used for amplifying CpG island DNA fragments of candidate methylation HR genes are listed in Table S3.

Immunohistochemical analysis

The status of hormone receptors HER2 and Ki-67/MIB-1 was determined by standard immunohistochemical analysis with DaKo Envision system (Dako, Denmark) and fluorescence *in situ* hybridization (FISH). The cutoff value for ERα -negative and PR-negative cases was 10%, and tumors with less than 10% expression were considered negative. For HER2 status, tumors that were immunohistochemically scored as (0+) (1+), or (2+) and were FISH negative were regarded as HER2 negative (no amplification). In the HER2-negative cases analyzed in the study, ERα -positive and/or PR-positive cases were defined as LBC, and ERα -negative and PR-negative cases were defined as TNBC.

Statistical analysis

All statistical analyses were performed using SPSS for Windows, version 12 (SPSS, Inc., Chicago, IL), and PRISM software for Windows, version 4 (GraphPad Prism, Inc., San Diego, CA). The methylation level (%) was analyzed as a continuous variable for the comparison of each gene with the sample's clinicopathologic features; mean and 95% confidence intervals (CIs) were calculated. Associations between continuous variables or the levels of DNA methylation and clinicopathologic variables were evaluated using analysis of variance (ANOVA) and Student's *t*-test. In parallel, we computed the median DNA methylation value and range for each sample, and we defined the receiver operating characteristic (ROC) curve (AUC) in SPSS software. *Z*-score analysis was used to normalize the methylation levels of several genes in each sample. The *Z*-score for each gene was calculated as follows: *Z*-score = (methylation level of each sample − mean value of methylation level)/standard deviation of methylation level. In this analysis, a *Z*-score greater than 0 means that the methylation level is greater than the mean value for the population. All reported *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

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References

Alvarez, S., Diaz-Uriarte, R., Osorio, A., Barroso, A., Melchor, L., Paz, M.F., Honrado, E., Rodríguez, R., Urioste, M., Valle, L., Díez, O., Cigudosa, J.C., Dopazo, J., Esteller, M. & Benitez, J. (2005) A predictor based on the somatic genomic changes of the BRCA1/BRCA2 breast cancer tumors identifies the non-BRCA1/BRCA2 tumors with BRCA1 promoter hypermethylation. *Clin. Cancer Res.* **11**, 1146–1153.

Audeh, M.W., Carmichael, J., Penson, R.T., Friedlander, M., Powell, B., Bell-McGuinn, K.M., Scott, C., Weitzel, J.N., Oaknin, A., Loman, N., Lu, K., Schmutzler, R.K., Matulonis, U., Wickens, M. & Tutt, A. (2010) Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: A proof-of-concept trial. *Lancet* **376**, 245–251.

Branham, M.T., Marzese, D.M., Laurito, S.R., Gago, F.E., Orozco, J.I., Tello, O.M., Vargas-Roig, L.M. & Roqué, M. (2012) Methylation profile of triple-negative breast carcinomas. *Oncogenesis* **1**, e17.

Byrski, T., Huzarski, T., Dent, R., Gronwald, J., Zuziak, D., Cybulski, C., Kladny, J., Gorski, B., Lubinski, J. & Narod, S.A. (2009) Response to neoadjuvant therapy with cisplatin in BRCA1-positive breast cancer patients. *Breast Cancer Res. Treat.* **115**, 359–363.

Cantor, S.B., Bell, D.W., Ganesan, S., Kass, E.M., Drapkin, R., Grossman, S., Wahrer, D.C., Sgroi, D.C., Lane, W.S., Haber, D.A. & Livingston, D.M. (2001) BACH1, a novel helicase-like protein, interacts directly with BRCA1 and contributes to its DNA repair function. *Cell* **105**, 149–160.

Catteau, A., Harris, W.H., Xu, C.F. & Solomon, E. (1999) Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene* **18**, 1957–1965.

Cerne, J.Z., Zong, L., Jelinek, J., Hilsenbeck, S.G., Wang, T., Oesterreich, S. & McGuire, S.E. (2012) BRCA1 promoter methylation status does not predict response to tamoxifen in sporadic breast cancer patients. *Breast Cancer Res. Treat.* **135**, 135–143.

Chen, Y., Zhou, J., Xu, Y., Li, Z., Wen, X., Yao, L., Xie, Y. & Deng, D. (2009) BRCA1 promoter methylation associated with poor survival in Chinese patients with sporadic breast cancer. *Cancer Sci.* **100**, 1663–1667.

Ciccia, A. & Elledge, S.J. (2010) The DNA damage response: making it safe to play with knives. *Mol. Cell* **40**, 179–204.

Collins, N., Wooster, R. & Stratton, M.R. (1997) Absence of methylation of CpG dinucleotides within the promoter of the breast cancer susceptibility gene BRCA2 in normal tissues and in breast and ovarian cancers. *Br. J. Cancer* **76**, 1150–1156.

Cucer, N., Taheri, S., Ok, E. & Ozkul, Y. (2008) Methylation status of CpG islands at sites -59 to +96 in exon 1 of the BRCA2 gene varies in mammary tissue among women with sporadic breast cancer. *J. Genet.* **87**, 155–158.

Dobrovic, A. & Simpfendorfer, D. (1997) Advances in Brief Methylation of the BRCAJ Gene in Sporadic Breast Cancer. *Cancer Res.* **57**, 3347–3350.

Esteller, M., Fraga, M.F., Guo, M. *et al.* (2001) DNA methylation patterns in hereditary human cancers mimic sporadic tumorigenesis. *Hum. Mol. Genet.* **10**, 3001–3007.

Esteller, M., Silva, J.M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I.C., Repasky, E.A., Gabrielson, E., Schutte, M., Baylin, S.B. & Herman, J.G. (2000) Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors. *J. Natl. Cancer Inst.* **92**, 564–569.

Heikkinen, K., Rapakko, K., Karppinen, S.M., Erkko, H., Knuutila, S., Lundán, T., Mannermaa, A., Børresen-Dale, A.L., Borg, A., Barkardottir, R.B., Petrini, J. & Winqvist, R. (2006) RAD50 and NBS1 are breast cancer susceptibility genes associated with genomic instability. *Carcinogenesis* **27**, 1593–1599.

Hsu, N.C., Huang, Y.F., Yokoyama, K.K., Chu, P.Y., Chen, F.M. & Hou, M.F. (2013) Methylation of BRCA1 Promoter Region Is Associated with Unfavorable Prognosis in Women with Early-Stage Breast Cancer. *PLoS ONE* **8**, e56256.

Jönsson, G., Staaf, J., Vallon-Christersson, J. *et al.* (2010) Genomic subtypes of breast cancer identified by array-comparative genomic hybridization display distinct molecular and clinical characteristics. *Breast Cancer Res.* **12**, R42.

Litman, R., Peng, M., Jin, Z., Zhang, F., Zhang, J., Powell, S., Andreassen, P.R. & Cantor, S.B. (2005) BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCJ. *Cancer Cell* **8**, 255–265.

Livingston, D.M. & Silver, D.P. (2008) Cancer: crossing over to drug resistance. *Nature* **451**, 1066–1067.

Magdinier, F., Ribieras, S., Lenoir, G.M., Frappart, L. & Dante, R. (1998) Down-regulation of BRCA1 in human sporadic breast cancer; analysis of DNA methylation patterns of the putative promoter region. *Oncogene* **17**, 3169–3176.

Margeli, M., Cirauqui, B., Castella, E., Tapia, G., Costa, C., Gimenez-Capitan, A., Barnadas, A., Sanchez Ronco, M., Benlloch, S., Taron, M. & Rosell, R. (2010) The Prognostic Value of BRCA1 mRNA Expression Levels Following Neoadjuvant Chemotherapy in Breast Cancer. *PLoS ONE* **5**, e9499.

Matros, E., Wang, Z.C., Lodeiro, G., Miron, A., Iglehart, J.D. & Richardson, A.L. (2005) BRCA1 promoter methylation in sporadic breast tumors: relationship to gene expression profiles. *Breast Cancer Res. Treat.* **91**, 179–186.

Meindl, A., Hellebrand, H., Wiek, C. *et al.* (2010) Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat. Genet.* **42**, 410–414.

Moelans, C.B., Verschuur-Maes, A.H.J. & Van Diest, P.J. (2011) Frequent promoter hypermethylation of BRCA2, CDH13, MSH6, PAX5, PAX6 and WT1 in ductal carcinoma *in situ* and invasive breast cancer. *J. Pathol.* **225**, 222–231.

Moskwa, P., Buffa, F.M., Pan, Y., Panchakshari, R., Gottipati, P., Muschel, R.J., Beech, J., Kulshrestha, R., Abdelmohsen, K., Weinstock, D.M., Gorospe, M., Harris, A.L., Helleday, T. & Chowdhury, D. (2011) miR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors. *Mol. Cell* **41**, 210–220.

Niwa, Y., Oyama, T. & Nakajima, T. (2000) BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers. *Jpn. J. Cancer Res.* **91**, 519–526.

Ohta, T., Sato, K. & Wu, W. (2011) The BRCA1 ubiquitin ligase and homologous recombination repair. *FEBS Lett.* **585**, 2836–2844.

Potapova, A., Hoffman, A.M., Godwin, A.K., Al-Saleem, T. & Cairns, P. (2008) Promoter hypermethylation of the PALB2 susceptibility gene in inherited and sporadic breast and ovarian cancer. *Cancer Res.* **68**, 998–1002.

Rahman, N., Seal, S., Thompson, D. *et al.* (2007) PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nat. Genet.* **39**, 165–167.

Renwick, A., Thompson, D., Seal, S. *et al.* (2006) ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nat. Genet.* **38**, 873–875.

Rice, J.C., Ozcelik, H., Maxeiner, P., Andrulis, I. & Futscher, B.W. (2000) Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. *Carcinogenesis* **21**, 1761–1765.

Sorlie, T., Tibshirani, R., Parker, J. *et al.* (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. *Proc. Natl Acad. Sci. USA* **100**, 8418–8423.

Steffen, J., Varon, R., Mosor, M. *et al.* (2004) Increased cancer risk of heterozygotes with NBS1 germline mutations in Poland. International journal of cancer. *Int. J. Cancer* **111**, 67–71.

Treilleux, I., Chapot, B., Goddard, S., Pisani, P., Angèle, S. & Hall, J. (2007) The molecular causes of low ATM protein expression in breast carcinoma; promoter methylation and levels of the catalytic subunit of DNA-dependent protein kinase. *Histopathol* **51**, 63–69.

Turner, N.C., Reis-Filho, J.S., Russell, A.M., Springall, R.J., Ryder, K., Steele, D., Savage, K., Gillett, C.E., Schmitt, F.C., Ashworth, A. & Tutt, A.N. (2007) BRCA1 dysfunction in sporadic basal-like breast cancer. *Oncogene* **26**, 2126–2132.

Tutt, A., Robson, M., Garber, J.E., Domchek, S.M., Audeh, M.W., Weitzel, J.N., Friedlander, M., Arun, B., Loman, N., Schmutzler, R.K., Wardley, A., Mitchell, G., Earl, H., Wickens, M. & Carmichael, J. (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.

Vo, Q.N., Kim, W.J., Cvitanovic, L., Boudreau, D.A., Ginzinger, D.G. & Brown, K.D. (2004) The ATM gene is a target for epigenetic silencing in locally advanced breast cancer. *Oncogene* **23**, 9432–9437.

Watanabe, Y., Kim, H.S., Castoro, R.J., Chung, W., Estecio, M.R., Kondo, K., Guo, Y., Ahmed, S.S., Toyota, M., Itoh, F., Suk, K.T., Cho, M.Y., Shen, L., Jelinek, J. & Issa, J.P. (2009) Sensitive and specific detection of early gastric cancer with DNA methylation analysis of gastric washes. *Gastroenterology* **136**, 2149–2158.

Wei, M., Grushko, T.A., Dignam, J., Hagos, F., Nanda, R., Sveen, L., Xu, J., Fackenthal, J., Tretiakova, M., Das, S. & Olopade, O.I. (2005) BRCA1 promoter methylation in sporadic breast cancer is associated with reduced BRCA1 copy number and chromosome 17 aneusomy. *Cancer Res.* **65**, 10692–10699.

Wei, M., Xu, J., Dignam, J., Nanda, R., Sveen, L., Fackenthal, J., Grushko, T.A. & Olopade, O.I. (2008) Estrogen receptor alpha, BRCA1, and FANCF promoter methylation occur in distinct subsets of sporadic breast cancers. *Breast Cancer Res. Treat.* **111**, 113–120.

Xu, X., Gammon, M.D., Zhang, Y., Bestor, T.H., Zeisel, S.H., Wetmur, J.G., Wallenstein, S., Bradshaw, P.T., Garbowski, G., Teitelbaum, S.L., Neugut, A.I., Santella, R.M. & Chen, J. (2009) BRCA1 promoter methylation is associated with increased mortality among women with breast cancer. *Breast Cancer Res. Treat.* **115**, 397–404.

Xu, Y., Diao, L., Chen, Y., Liu, Y., Wang, C., Ouyang, T., Li, J., Wang, T., Fan, Z., Fan, T., Lin, B., Deng, D., Narod, S.A. & Xie, Y. (2013) Promoter methylation of BRCA1 in triple-negative breast cancer predicts sensitivity to adjuvant chemotherapy. *Annals Oncol.* **24**, 1498–1505.

Zhang, F., Ma, J., Wu, J., Ye, L., Cai, H., Xia, B. & Yu, X. (2009) PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr. Biol.* **19**, 524–529.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1 Aberrant DNA methylation status of HR genes in cancer cell lines

Table S2 Aberrant DNA methylation status of HR genes in breast cancer tissues

Table S3 Sequence and the position of primers used in the study

Figure S1 An example pyro-gram data for the sequence of bisulfite-treated DNA in 6 final candidate genes (BRCA1, BRCA2, BARD1, MDC1, ABRA1 and PALB2).

Figure S2 Summary of the process for selection of the genes to be addressed in the current study.

Figure S3 The sequence and position of the primers used in the study. Black bars with arrows indicate the coding region of HR genes.

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Article

A DNA-Damage Selective Role for BRCA1 E3 Ligase in Claspin Ubiquitylation, CHK1 Activation, and DNA Repair

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Summary

Background: The breast and ovarian cancer suppressor BRCA1 is essential for cellular responses to DNA damage [1]. It heterodimerizes with BARD1 to acquire an E3 ubiquitin (Ub) ligase activity that is often compromised by cancer-associated mutations [2]. Neither the significance of this activity to damage responses, nor a relevant in vivo substrate, is clear.

Results: We have separated DNA-damage responses requiring the BRCA1 E3 ligase from those independent of it, using a gene-targeted point mutation in vertebrate DT40 cells that abrogates BRCA1's catalytic activity without perturbing BARD1 binding. We show that BRCA1 ubiquitylates claspin, an essential coactivator of the CHK1 checkpoint kinase, after topoisomerase inhibition, but not DNA crosslinking by mitomycin C. BRCA1 E3 inactivation decreases chromatin-bound claspin levels and impairs homology-directed DNA repair by interrupting signal transduction from the damage-activated ATR kinase to its effector, CHK1.

Conclusions: Our findings identify claspin as an in vivo substrate for the BRCA1 E3 ligase and suggest that its modification selectively triggers CHK1 activation for the homology-directed repair of a subset of genotoxic lesions. This mechanism unexpectedly defines an essential but selective function for BRCA1 E3 ligase activity in cellular responses to DNA damage.

Introduction

There is abundant evidence that the breast and ovarian tumor suppressor, BRCA1, has essential functions in diverse cellular processes implicated in the DNA damage response [1]. How BRCA1 mediates these functions remains unclear. Much attention has focused on the E3 ubiquitin ligase activity acquired when BRCA1 heterodimerizes through its amino-terminal RING domain with an evolutionarily conserved RING partner, BARD1 [3]. Deleterious missense mutations affecting the BRCA1 RING domain can be found in familial breast cancers that do not vitiate BARD1 binding but can nevertheless abolish E3 ligase activity [2, 4], suggestive of its functional importance. However, a BRCA1 point mutation abolishing E3 activity does not impair ionizing radiation sensitivity or the repair of endonuclease-induced DNA breaks by homologous recombination [5]. Furthermore, mice harboring the mutation do not develop breast cancers [6]. On the other hand, inactivation of BRCA1 E3 ligase activity can derepress heterochromatic satellite DNA transcription, an event that has been linked to abnormalities in DNA repair and mitosis [7]. Thus, if and how BRCA1's E3 ligase activity is necessary for the cellular response to DNA damage remains uncertain.

BRCA1 has been implicated in several processes essential for the cellular response to DNA lesions repaired by homologous recombination [1]. One key step involves activation of the checkpoint kinase CHK1, an essential component of the damage response machinery in vertebrates. Phosphorylation of CHK1 occurs on chromatin. The phosphorylated CHK1 is released from chromatin and the dissociation is required for checkpoint activation [8]. BRCA1 has been implicated in CHK1 activation via ATR and claspin. ATR-phosphorylated claspin forms a trimolecular complex containing BRCA1 and CHK1 [9]. Complex formation is essential for CHK1 phosphorylation and activation [9]; in turn, activated CHK1 mediates checkpoint enforcement as well as DNA repair by homologous recombination [10, 11], through modulation of the interaction between BRCA2 and the recombinase RAD51 [12, 13]. However, the mechanism through which BRCA1 controls the claspin-CHK1 axis, and in particular, whether its E3 ligase activity is required, is not known.

Here, we have used "hit-and-run" gene targeting to create a DT40 cell line carrying a single mutation in BRCA1, replacing Val26 in the RING domain with Ala (V26A). The V26A mutation abrogates E3 ligase activity without affecting the interaction between BRCA1 and BARD1. Unexpectedly, E3 inactivation reveals a mechanism distinguishing DNA damage responses that require BRCA1's catalytic activity, from those independent of it. Whereas the BRCA1 E3 ligase is essential for chromatin association of claspin and CHK1 activation when a topoisomerase poison, camptothecin (CPT), blocks DNA replication, it is dispensable for these events after replication blockage by a DNA crosslinker, mitomycin C (MMC). In turn, downstream responses regulated by CHK1, including RAD51 foci formation, sister chromatid exchange, and cellular sensitivity, are selectively impaired after exposure to CPT, but not MMC. Thus, our work suggests that the BRCA1 E3 ligase selectively triggers claspin-CHK1 activation, providing the first example of an E3-dependent mechanism underlying an essential function for BRCA1 in the cellular response to DNA damage.

Results and Discussion

BRCA1 has been implicated in the cellular response to DNA double-strand breaks leading to their resolution by homology-directed repair (HDR) [1]. We used avian DT40 cells, a well-established model widely used to study the role of BRCA1 in this response [14–16], to determine the functional

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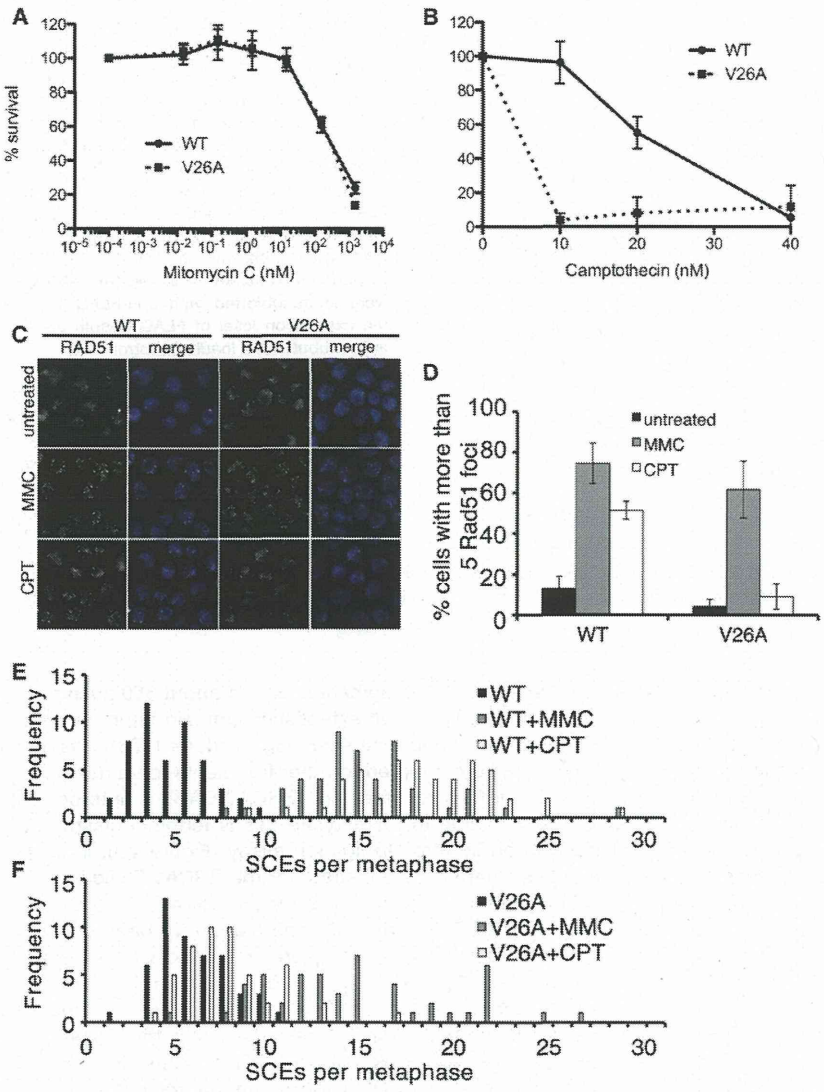

Figure 1. The V26A Point Mutation Defines a Subset of Genotoxic Responses Requiring BRCA1 E3 Ligase Activity

(A and B) Cell viability, as determined by the CellTiter Blue colorimetric assay, is expressed as a percentage of the untreated control sample for both cell lines. The percentage of viable cells is plotted for each of the indicated doses of mitomycin C (MMC) (A) and camptothecin (CPT) (B). Error bars (some too small to be visible) show the SD from the mean of four independent wells from three independent experiments.

(C) Representative micrographs of WT or V26A DT40 cells, undamaged (top row) or treated for 6 hr with the indicated drugs (rows 2–4) and stained with anti-RAD51 (grayscale). DNA staining was with DAPI (blue).

(D) Histogram showing the percentage of cells with >5 RAD51 foci. RAD51 foci were counted in WT and V26A cells (n = 100) before and after exposure to the indicated drugs (MMC: 150 nM for 8 hr, CPT: 100 nM for 8 hr). Error bars show the SD from the mean of three independent experiments.

(E and F) Histograms showing the frequency distribution for spontaneous, MMC (150 nM for 8 hr) or CPT (100 nM for 8 hr)-induced sister chromatid exchanges (SCE's) in WT (E) or V26A DT40 cells (F). Spontaneous SCE formation is intact, but only MMC (gray color bars) induces SCEs in V26A mutant cells. See also Figure S1 and Table S1.

significance of BRCA1 E3 ligase activity. In mammalian BRCA1 homologs, Ile26 in the RING domain is implicated in binding to E2 ubiquitin conjugating enzymes [2]. Its substitution by Ala abolishes BRCA1's E3 ligase activity without affecting heterodimerization to BARD1 [2]. Val26 in the avian RING domain conservatively replaces Ile26 in its mammalian counterparts, reflecting their strong evolutionary conservation (see Figure S1A available online). We cloned the RING domains of avian BRCA1 and BARD1 from a DT40 complementary DNA (cDNA) library and introduced the Val26Ala (V26A) point mutation by site-directed mutagenesis. When cotransfected into 293T cells with the avian BARD1 RING domain, the V26A mutant form of the *Gg*BRCA1 RING domain still binds to the BARD1 RING domain (Figure S1B) but fails to catalyze the transfer of ubiquitin in vitro (Figure S1C). Thus, replacement of Val26 with Ala in avian BRCA1 abolishes the E3 ligase activity of the RING domain but not its interaction with BARD1. This confirms that avian Val26 is functionally cognate with Ile26 in mammalian BRCA1.

Figure S1D shows the gene-targeting strategy used to create a DT40 cell line carrying a Val26-Ala substitution in the endogenous *BRCA1* gene. The targeting vector carries a blasticidin or neomycin resistance cassette flanked by *loxP* sites for the CRE recombinase, enabling recycling of the resistance marker by CRE transfection between sequential rounds of targeted integration. Homozygous Val26-Ala replacement was confirmed by amplification of the targeted *BRCA1* genomic locus using the indicated primers (Figure S1D), by a diagnostic restriction digest with BsrDI (Figure S1E) and by nucleotide sequencing (data not shown). Western blotting of extracts from BRCA1 wild-type (WT) or BRCA1$^{V26A/V26A}$ (V26A) cells confirmed that the WT and mutant BRCA1 proteins were expressed at similar levels (Figures S1F and S1G). Thus, in accord with previous reports [14, 15], BRCA1 E3 ligase is dispensable for the viability and growth of DT40 cells.

BRCA1 has been implicated in the cellular response to agents that inflict DNA lesions repaired by HDR. These include the topoisomerase I poison, camptothecin (CPT), which triggers DNA breakage at replication forks [17], or the DNA crosslinking agent, mitomycin C (MMC), which creates intrastrand lesions as well as interstrand crosslinks that prevent the movement of replication forks [18]. We exposed WT or V26A cells to increasing doses of CPT or MMC for 40 hr before measuring cell viability (Figures 1A and 1B). V26A cells exhibit enhanced sensitivity to CPT, but not MMC. This raises the unexpected possibility that BRCA1 E3 ligase activity is selectively required for certain cellular responses leading to the repair of certain types of DNA lesions by HDR, but not others.

Two key cellular events report on the integrity of HDR in DT40 cells. First, inactivating mutations in known HDR