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Annals of Oncology 29: 654–660, 2018
doi:10.1093/annonc/mdx821
Published online 23 December 2017

ORIGINAL ARTICLE

Impact of homologous recombination deficiency biomarkers on outcomes in patients with triple-negative breast cancer treated with adjuvant doxorubicin and cyclophosphamide (SWOG S9313)

P. Sharma[1*], W. E. Barlow[2,3], A. K. Godwin[1], H. Pathak[1], K. Isakova[1], D. Williams[4], K. M. Timms[4], A. R. Hartman[4], R. J. Wenstrup[4], H. M. Linden[5,6], D. Tripathy[7], G. N. Hortobagyi[7] & D. F. Hayes[8]

[1]Division of Medical Oncology, Department of Internal Medicine, University of Kansas Medical Center, Kansas City; [2]SWOG Statistical Center, Seattle; [3]Cancer, Research and Biostatistics (CRAB), Seattle; [4]Myriad Genetics, Inc., Salt Lake City; [5]Department of Medicine, University of Washington, Seattle; [6]Seattle Cancer Care Alliance, Seattle; [7]Department of Breast Medical Onocolgy, The University of Texas MD Anderson Cancer Center, Houston; [8]Department of Internal Medicine, University of Michigan, Ann Arbor, USA

*Correspondence to: Dr Priyanka Sharma, Division of Medical Oncology, Department of Internal Medicine, University of Kansas Medical Center, 2330 Shawnee Mission Parkway, Suite 210, MS 5003, Westwood, KS 66205, USA. Tel: +1-913-588-6029; E-mail: psharma2@kumc.edu

Background: Homologous recombination deficiency (HRD)-causing alterations have been reported in triple-negative breast cancer (TNBC). We hypothesized that TNBCs with HRD alterations might be more sensitive to anthracycline plus cyclophosphamide-based chemotherapy and report on HRD status and *BRCA1* promoter methylation (PM) as prognostic markers in TNBC patients treated with adjuvant doxorubicin (A) and cyclophosphamide (C) in SWOG9313.

Patients and methods: In total, 425 TNBC patients were identified from S9313. HRD score, tumor *BRCA1/2* sequencing, and *BRCA1* PM were carried out on DNA isolated from formalin-fixed paraffin-embedded tissue. Positive HRD status was defined as either a deleterious tumor *BRCA1/2* (t*BRCA*) mutation or a pre-defined HRD score ≥42. Markers were tested for prognostic value on disease-free survival (DFS) and overall survival (OS) using Cox regression models adjusted for treatment assignment and nodal status.

Results: HRD status was determined in 89% (379/425) of cases. Of these, 67% were HRD positive (27% with t*BRCA* mutation, 40% t*BRCA*-negative but HRD score ≥42). HRD-positive status was associated with a better DFS [hazard ratio (HR) 0.72; 95% confidence interval (CI) 0.51–1.00; $P = 0.049$] and non-significant trend toward better OS (HR = 0.71; 95% CI 0.48–1.03; $P = 0.073$). High HRD score (≥42) in t*BRCA*-negative patients ($n = 274$) was also associated with better DFS (HR = 0.64; 95% CI 0.43–0.94; $P = 0.023$) and OS (HR = 0.65; 95% CI 0.42–1.00; $P = 0.049$). *BRCA1* PM was evaluated successfully in 82% (348/425) and detected in 32% of cases. The DFS HR for *BRCA1* PM was similar to that for HRD but did not reach statistical significance (HR = 0.79; 95% CI 0.54–1.17; $P = 0.25$).

Conclusions: HRD positivity was observed in two-thirds of TNBC patients receiving adjuvant AC and was associated with better DFS. HRD status may identify TNBC patients who receive greater benefit from AC-based chemotherapy and should be evaluated further in prospective studies.

Clinical Trials Number: Int0137 (The trial pre-dates Clinicaltrial.Gov website establishment)

Key words: triple negative breast cancer, homologous recombination deficiency (HRD), biomarker, chemotherapy, *BRCA* mutation

Introduction

Adjuvant chemotherapy reduces the risk of distant recurrence and death in patients with triple-negative breast cancer (TNBC). Even so, approximately 20%–40% of patients with early stage TNBC develop metastatic disease [1–3]. The dearth of reliable response/resistance biomarkers for standard chemotherapy has

slowed the development of newer agents for TNBC. Ideally, robust tumor biomarker tests would provide insight into which TNBC patients are likely to do well with anthracycline/cyclophosphamide (AC)-based adjuvant chemotherapy or, alternatively, may provide insight into mechanisms of resistance to this strategy and identification of alternative treatment approaches.

Homologous recombination (HR) is a DNA repair mechanism responsible for repair of double-strand breaks (DSBs). *BRCA1/2* genes, along with other Fanconi anemia (FA) pathway genes (*RAD51D*, *NBN*, *ATM*, etc.), are key components of HR-mediated DNA repair. Germline *BRCA1/2* mutations are prototypic molecular alterations that confer HR deficiency (HRD) and sensitivity to DNA-damaging therapy [4, 5].

Inherited and acquired defects in HR might serve as response biomarkers or as therapeutic targets in breast cancer. To this end, development and clinical evaluation of platforms to identify HR defects are of interest, especially in TNBC, as this subtype is considered enriched for HR pathway deficiency [6–8]. Approximately 10%–20% of TNBC patients harbor germline *BRCA1/2* mutations, and another 3%–5% demonstrate somatic *BRCA1/2* mutations [9, 10]. However, DNA repair capacity in the tumor may be altered through other mechanisms, such as somatic or germline mutation in other FA pathway genes, DNA methylation or attenuated mRNA expression. Hypermethylation of the *BRCA1* promoter has been proposed as one of the mechanisms for silencing *BRCA1* expression in sporadic TNBC, and this epigenetic inactivation of *BRCA1* is associated with a gene expression profile similar to that of inherited *BRCA1* mutation-associated breast cancer [9, 11–13]. Employing more global measures, rather than relying on documented changes in specific genes, may identify more patients with HR deficiency. The HRD score is an algorithmic assessment of three measures of tumor genomic instability (loss of heterozygosity, telomeric allelic imbalance, and large-scale state transitions) [6, 14, 15]. High HRD scores have been shown to be significantly associated with defects in *BRCA1/2* and are associated with sensitivity to neoadjuvant platinum-based chemotherapy in TNBC [6, 16, 17].

We postulated that DNA repair deficiency phenotype can be caused and measured in different ways and could affect response to DNA-damaging or repair-inhibiting therapies like doxorubicin (which induces DNA DSBs) and cyclophosphamide (an alkylating agent which causes DNA crosslinks leading to DSBs). In this study, we sought to determine whether the HRD score and other related markers are prognostic in early stage TNBC patients who participated in SWOG adjuvant trial S9313 (Intergroup Protocol 0137). We hypothesized that HRD status and *BRCA1* promoter methylation (PM) would be prognostic in TNBC patients treated with adjuvant AC.

Methods

Patients

Patient selection, assay performance, and data analysis are reported according to the REMARK criteria [18]. Breast tumor specimens prepared from paraffin blocks collected prospectively from S9313 participants were used for this study. In S9313, patients with either high-risk node-negative or low-risk node-positive breast cancer were randomly assigned to one of two equivalent dose schedules of doxorubicin (A) and cyclophosphamide (C) chemotherapy [19]. There was no difference in disease-free survival (DFS) or overall survival (OS) for patients treated on the two arms [19]. Details of the study population and treatment schedule are provided in the supplementary, available at *Annals of Oncology* online.

Estrogen receptor (ER) and progesterone receptor (PR) were determined both locally and centrally (Allred scoring method; ER and PR Allred score of 0 was considered negative). Human epidermal growth factor receptor 2 (HER2) was determined centrally by FISH and immunohistochemistry [20]. TNBC was defined as ER- and PR negative (on both local and central review) and HER2-negative in accordance with the 2013 ASCO-CAP HER2 testing guidelines [21]. Laboratories performing the biomarkers were blinded to patient clinical and outcome information.

Tissue processing. Genomic DNA and RNA were isolated using standard techniques and commercially available kits in research laboratory according to CLIA protocol (described in supplementary, available at *Annals of Oncology* online).

HRD status. Custom enrichment panel and next-generation sequencing were used to generate genome-wide single nucleotide polymorphism profiles from which the three components of the HRD score are calculated [6]. The panel also includes probes targeting the complete coding region of *BRCA1* and *BRCA2*. A detailed description of the assay panel design, sequence alignment, and mutation detection methods has been published previously [6]. Mutations were only included in the analysis if classified as deleterious or suspected deleterious. HRD status was classified as positive if there was either a mutated tumor *BRCA1/2* or a predefined HRD score ≥ 42 [17] HRD was classified as negative if HRD score was < 42 and tumor lacked deleterious *BRCA1/2* mutation. HRD status could not be determined if HRD assay failed and tumor *BRCA1/2* analysis was either negative or failed [17]. Additional details are provided in the supplementary Material, available at Annals of Oncology online.

BRCA1 *PM.* *BRCA1* PM was assessed following bisulfite conversion of genomic DNA followed by methylation-specific PCR and agarose electrophoresis as described previously [13]. The presence of a methylated band was recorded as 'positive' for *BRCA1* PM.

BRCA1 *gene expression.* *BRCA1* expression was measured using NanoString Technologies gene expression assays, following the manufacturer's protocol.

Statistical analyses. Disease-free survival was defined as the time from registration to first invasive recurrence (local, regional, or distant), to new primary cancer in the contralateral breast, or to death due to any cause. OS was defined as time from registration to death from any cause. Patients were censored on the date of last contact if an event had not been observed. Survival curves were assessed by the Kaplan–Meier method and unadjusted survival comparisons conducted using log-rank tests. The markers were tested for prognostic effect on DFS and OS using a Cox regression model with adjustment for randomized treatment assignment and nodal status. All reported *P*-values and confidence intervals (CIs) are from two-sided tests.

Results

Identification of the study population

The selection process of the 425 TNBC samples from S9313 is provided in supplementary Figure S1, available at *Annals of Oncology* online. We have reported previously that the DFS and

Table 1. Biomarkers and outcomes

Biomarker	*n* (%)	5-year DFS (95% CI)	5-year OS (95% CI)	10-year DFS (95% CI)	10-year OS (95% CI)
HRD status ($N = 379$)					
Negative[a]	124 (33)	65.2% (56.0% to 72.8%)	76.5% (68.0% to 83.0%)	58.3% (49.0% to 66.5%)	66.4% (57.2% to 74.0%)
Positive[b]	255 (67)	78.7% (73.2% to 83.3%)	85.8% (80.9% to 89.6%)	70.5% (64.4% to 75.7%)	77.5% (71.8% to 82.3%)
HRD score in *BRCA1/2* wild-type ($N = 274$)					
<42	124 (45)	65.2% (56.0% to 72.8%)	76.5% (68.0% to 83.0%)	58.3% (49.0% to 66.5%)	66.4% (57.2% to 74.0%)
≥42	150 (55)	80.5% (73.2% to 86.1%)	85.2% (78.5% to 90.0%)	74.4% (66.6% to 80.7%)	79.7% (72.2% to 85.3%)
BRCA1 PM ($N = 348$)					
Present	111 (32)	76.4% (67.3% to 83.3%)	80.9% (72.3% to 87.1%)	70.8% (61.3% to 78.4%)	77.1% (68.0% to 83.9%)
Absent	237 (68)	73.8% (67.7% to 78.9%)	82.6% (77.2% to 86.9%)	64.5% (58.0% to 70.3%)	72.2% (65.9% to 77.5%)

CI, confidence interval; DFS, disease-free survival; HRD, homologous recombination deficiency; OS, overall survival.
[a]HRD negative status=HRD score < 42 and absence of tumor *BRCA* mutation.
[b]HRD positive status=HRD score ≥ 42 or presence of tumor *BRCA* mutation.

OS for patients with and without archived tissue specimens were similar in this trial [22].

Patient demographics

Demographic and clinical characteristics of the 425 TNBC patients are described in supplementary Table S1, available at *Annals of Oncology* online. Median age was 45 years, and 33% had lymph node-positive disease. At a median follow-up of 12.6 years, there were 166 DFS and 129 OS events.

Biomarker results availability

HRD status, which depends on both *BRCA* mutation status and HRD score, could be determined in 89% (379/425) of patients. *BRCA1* PM results were determined in 82% (348/425) of patients (see supplementary Figure S2, available at *Annals of Oncology* online, for details). There was no difference in DFS by HRD status known or not known (log-rank $P = 0.97$) or by *BRCA1* PM status known or unknown ($P = 0.86$). Similarly, there was no difference in OS by HRD status known or not known or by *BRCA1* PM status known or not known ($P = 0.75$ for both).

Association of HRD status with outcome

For patients with available HRD status results, 27% (105/379) demonstrated tumor *BRCA* mutation (*BRCA1* = 81, *BRCA2* = 23, *BRCA1* and *BRCA2* = 1) and another 40% (150/379) demonstrated HRD score ≥42 with wild-type *BRCA1/2*. Taken together, 67% (255/379) of patients had positive HRD status (HRD score ≥42 or presence of tumor *BRCA* mutation), and 33% (124/379) of patients had negative HRD status (HRD score <42 and absence of tumor *BRCA* mutation).

Positive HRD status was associated with a better DFS [hazard ratio (HR) = 0.72; 95% CI 0.51–1.00, $P = 0.049$] and non-significant trend toward better OS (HR = 0.71; 95% CI 0.48–1.03, $P = 0.073$), adjusting for treatment arms and nodal status (Table 1 and Figure 1A and B). We also considered whether the association of HRD status with outcomes was constant over the follow-up period. A test of the proportional hazards assumption of the Cox model suggested that all three covariate HRs (HRD status, treatment effect, and nodal status) varied over the long follow-up period. Restricting follow-up to the first 5 years showed a stronger impact of HRD status on DFS (HR = 0.57; 95% CI 0.38–0.85, $P = 0.006$) and non-significant trend toward better OS (HR = 0.63; 95% CI 0.38–1.03, $P = 0.064$). After the first 5 years, there was little impact of HRD status on DFS (HR = 1.21; 95% CI 0.65–2.28, $P = 0.55$) and OS (HR = 0.85; 95% CI 0.47–1.53, $P = 0.59$). Thus, the prognostic effect of HRD status in TNBC appeared to be more pronounced in the first 5 years.

Association of HRD score with outcome in patients with *BRCA1/2* wild-type tumors

Of the 274 patients with *BRCA1/2* wild-type tumors and known HRD score, 55% (150/274) demonstrated HRD score of ≥42. High HRD score (≥42) in patients with *BRCA1/2* wild-type tumors was associated with better DFS (HR = 0.64; 95% CI 0.43–0.94; $P = 0.023$) and OS (HR = 0.65; 95% CI 0.42–1.00; $P = 0.049$), adjusting for treatment and nodal status (Figure 1C and D and Table 1).

Association of tumor mutation status with outcome

Tumor *BRCA* mutation status was positive in 25% (105/412) of patients. Tumor *BRCA* mutation status (mutant or wild-type) did not impact DFS ($P = 0.59$) or OS ($P = 0.90$), adjusting for nodal status and treatment (Figure 2A and B).

Association of *BRCA1* PM with outcome

The presence of *BRCA1* PM was detected in 32% (111/348) of patients. Although the DFS HR for *BRCA1* PM was similar to that for HRD, it was not statistically significant (HR = 0.79; $P = 0.25$). OS had similar results (Figure 2C and D).

Association of *BRCA1* PM with *BRCA1* mRNA expression

BRCA1 mRNA expression results were available for 396/425 (87%) samples, and both *BRCA1* PM and *BRCA1* expression data

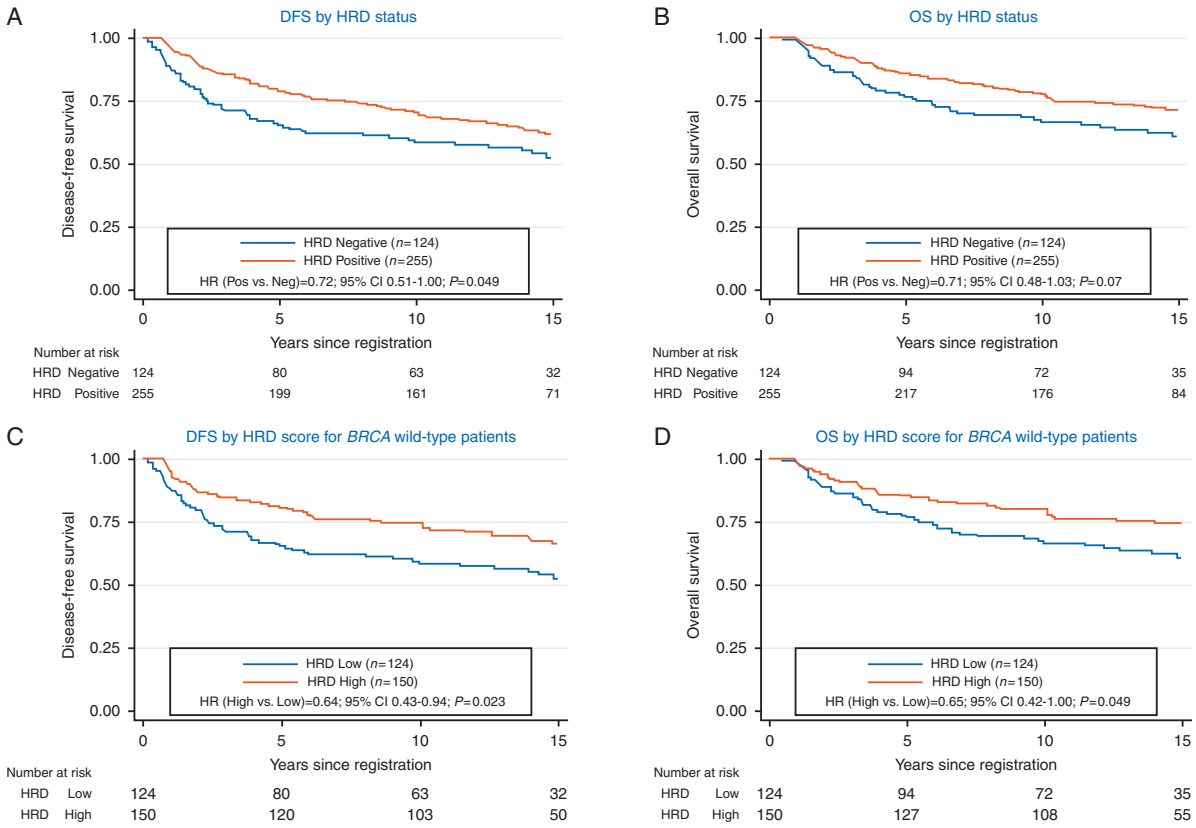

Figure 1. (A) Disease-free survival (DFS) by homologous recombination deficiency (HRD) status. (B) Overall survival (OS) by HRD status. (C) DFS by HRD score for *BRCA* wild-type patients. (D) OS by HRD score for *BRCA* wild-type patients.

were available from 330 samples. As expected, the presence of *BRCA1* PM was associated with lower *BRCA1* transcript expression (Wilcoxon $P < 0.0001$) (supplementary Figure S3, available at *Annals of Oncology* online).

Association of HRD score with tumor *BRCA1/2* mutation and *BRCA1* promoter methylation

Supplementary Table S2, available at *Annals of Oncology* online, provides the overlap between HRD score, *BRCA1* PM, and tumor *BRCA* mutation. Compared with tumors without *BRCA1/2* mutation, tumors with *BRCA1/2* mutation demonstrated higher HRD scores (median HRD score was 61 for tumors with *BRCA* mutation vs. 47 for tumors without *BRCA* mutation, $P < 0.0001$) (supplementary Figure S4A, available at *Annals of Oncology* online). Similarly, compared with tumors without *BRCA1* PM, tumors with *BRCA1* PM demonstrated higher HRD scores (median HRD score 66 for tumors with *BRCA1* PM versus 43 for tumors without *BRCA1* PM, $P < 0.0001$) (supplementary Figure S4B, available at *Annals of Oncology* online). *BRCA1/2* mutation and *BRCA1* PM collectively accounted for 83% (187/255) of patients with positive HRD status. There was very little overlap between *BRCA1/2* mutation and *BRCA1* PM. Out of 346 samples for which both *BRCA1/2* mutation and *BRCA1* PM data were available, only 3% ($n = 11$) demonstrated both mutation and methylation (all 11 demonstrated HRD score ≥ 42).

Discussion

In this study, we observed that two-thirds of TNBC patients treated with adjuvant AC in S9313 exhibited HRD positivity (based on the HRD score and tumor *BRCA* mutation). Patients with positive HRD status had better 10-year DFS compared to those with negative HRD status (HR = 0.72). The prognostic impact of HRD status was independent of nodal status and seemed to be more pronounced for the first 5 years (5-year DFS HR = 0.57). We further observed high HRD score (≥ 42) in more than half (55%) of t*BRCA* wild-type patients, and high HRD score in these patients was independently associated with better DFS (HR = 0.64) and OS (HR = 0.65), thus confirming that HR deficiency mediated by mechanisms other than *BRCA* mutation is present in a substantial proportion of TNBC and is likely to be biologically, and perhaps clinically, important.

Tumor *BRCA1/2* mutation was noted in 25% of our cohort. Due to lack of availability of germline DNA, we could not determine whether these mutations were germline or somatic in nature. However, this *BRCA* mutation prevalence is consistent with known literature. Previous studies have demonstrated germline *BRCA1/2* mutations in 15%–20% and somatic *BRCA1/2* mutations in 3%–5% of unselected TNBC [9, 10, 23]. Tumor *BRCA* mutation status was not prognostic in our cohort, (perhaps due to relatively modest number of patients with *BRCA* mutation), a finding which is also consistent with previous

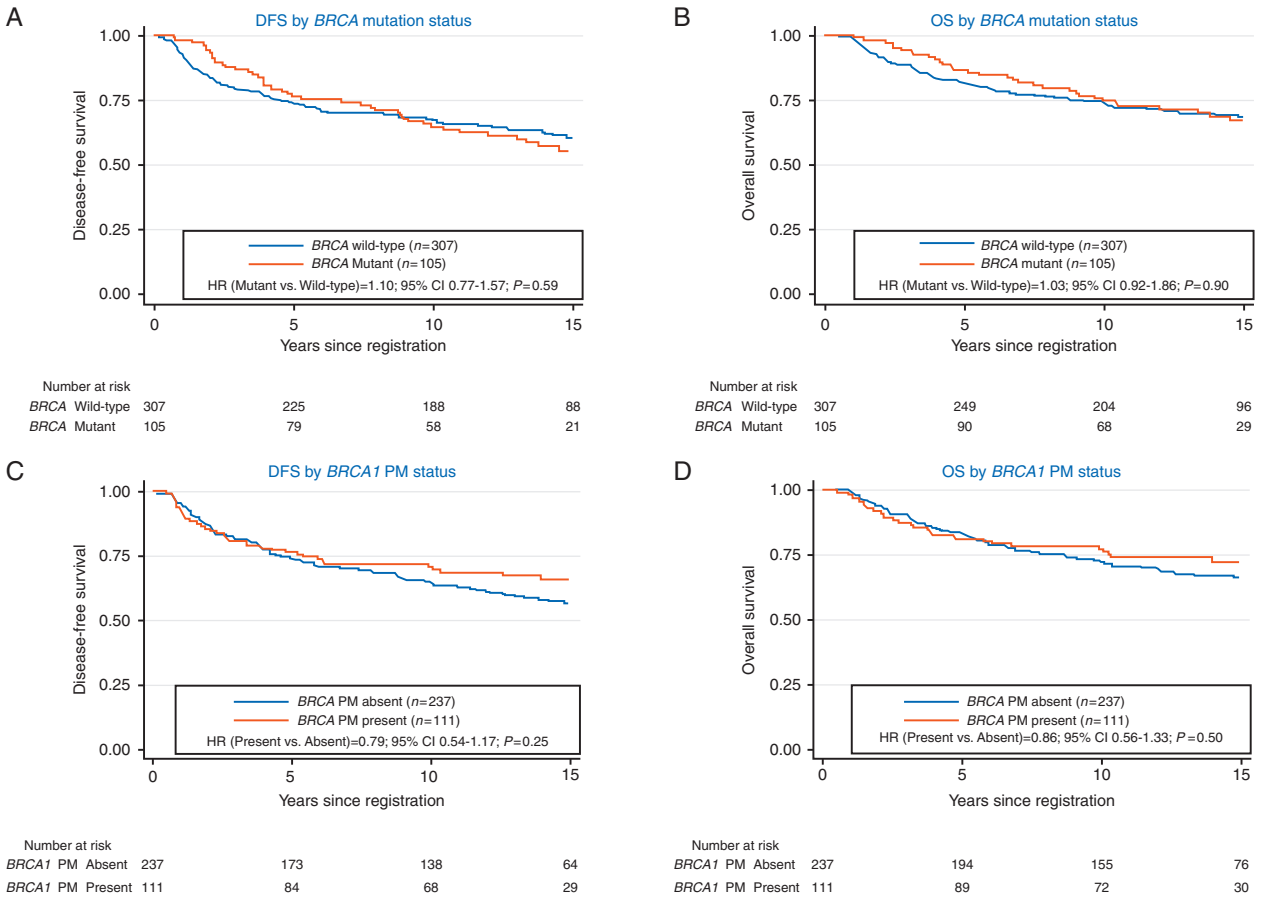

Figure 2. (A) Disease-free survival (DFS) by *BRCA* mutation status. (B) Overall survival (OS) by *BRCA* mutation status. (C) DFS by *BRCA1* promoter methylation (PM) status. (D) OS by *BRCA1* PM status. *BRCA* mutation information was available for 312 patients, and *BRCA1* PM was available for 348 subjects.

studies [24, 25]. Given the prevalence of *BRCA* mutation, this patient cohort was probably at high risk for other *BRCA*-related cancers. However, information on contralateral breast cancer or other non-breast cancer (e.g. ovarian) related deaths are not currently available, thus we cannot comment on the potential impact of these events on long-term DFS.

BRCA1 PM was associated with lower *BRCA1* mRNA expression (corresponding to epigenetic silencing of *BRCA1* gene) and was associated with higher HRD scores. Several prior studies have evaluated *BRCA1* PM in TNBC patients treated with various chemotherapy regimens, showing conflicting prognostic impact [13, 26, 27]. In this large, uniformly treated TNBC patient population, we did not observe a prognostic impact of *BRCA1* PM on outcome. There was a notable lack of overlap between *BRCA* mutation and *BRCA1* PM, supporting the notion that mechanisms of gene function loss appear to be non-redundant and invoke the principle of complementarity.

In an exploratory analysis, the combined effect of *BRCA* mutation and *BRCA1* PM on outcome was not found to be prognostic (data not shown). Thus, HRD score/status continues to be a more robust prognostic factor even if *BRCA* mutation and methylation status are known, indicating that *BRCA* mutation and methylation do not capture all of the patients with HR deficiency.

These data were derived from a mature, prospective randomized clinical trial, obviating concerns about bias in outcome ascertainment. Further, our results demonstrate that formalin-fixed paraffin-embedded tumor tissue collected > 20 years ago as part of an intergroup trial can be successfully used for DNA- and RNA-based biomarkers. However, the study does have certain limitations. All patients received adjuvant AC chemotherapy, without an untreated or alternatively treated comparator arm. Thus, we cannot determine whether HRD is prognostic in spite of or predictive of benefit from AC chemotherapy. Furthermore, we cannot remark on whether HRD would predict benefit from taxanes, which are currently part of all standard neo/adjuvant chemotherapy for breast cancer. Recent data do suggest that positive HRD status is associated with improved pathological complete response to neoadjuvant anthracycline plus taxane chemotherapy and also to platinum-based chemotherapy [17, 28]. Although we show that the prognosis of patients with high HRD is superior to those with low HRD, currently there are insufficient data to with hold or select other treatments, such as taxanes or platinum agents, based on HRD status.

In summary, HRD status is prognostic in TNBC patients who were uniformly treated with AC chemotherapy. The clinical utility of HRD in the presence of DNA-damaging therapy like anthracyclines, platinum salts, and poly(ADP-ribose)

polymerase (PARP) inhibitors is the subject of ongoing investigations. Neoadjuvant clinical trials (NCT01982448 and NCT02032277) are evaluating the ability of this HRD assay to predict pathological complete response with platinum, taxane, or AC/taxane chemotherapy in TNBC. SWOG S1416 (NCT02595905) is using multiple HRD biomarkers to predict benefit from addition of a PARP inhibitor to platinum chemotherapy in metastatic TNBC. Our study demonstrates the clinical validity of the HRD assay; additional studies are warranted to further refine and establish the clinical utility of HR deficiency in TNBC [29].

Acknowledgement

The authors wish to gratefully acknowledge the late Dr Robert Livingston for his important contributions to SWOG and to study S9313.

Funding

National Cancer Institute/National Clinical Trials Network grants U10CA180888, U10CA180819, U10CA180801, U10CA180858 and in part by Amgen, American Society of Clinical Oncology Advanced Clinical Cancer Research Award by Conquer Cancer Foundation (PS), the Eileen Stein Jacoby Fund, University of Kansas Cancer Center's Cancer Center Support Grant (P30 CA168524) Biospecimen Repository Core Facility, Breast Cancer Research Foundation (DFH and PS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Disclosure

DW reports employment by and ownership interest (stock) in Myriad Genetics, LLC. RW reports employment by, ownership interest (stock) in, and a leadership role in Myriad Genetics, LLC. KMT reports employment by, ownership interest (stock) in, and patents, royalties, other Intellectual Property, and other expenses from Myriad Genetics, LLC. A-RH reports employment by and ownership interest (stock) in Myriad Genetics, LLC. All remaining authors have declared no conflicts of interest.

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