Cancer Therapy: Clinical

Clinical Cancer Research

Intratumor Heterogeneity of Homologous Recombination Deficiency in Primary Breast Cancer

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Abstract

Purpose: The 3-biomarker homologous recombination deficiency (HRD) assay measures the number of telomeric allelic imbalances, loss of heterozygosity, and large-scale state transitions in tumor DNA and combines these metrics into a single score that reflects DNA repair deficiency. The goal of this study is to assess the consistency of these HRD measures in different biopsies from distinct areas of the same cancer.

Experimental Design: HRD scores, BRCA mutation status, and *BRCA1* promoter methylation were assessed in 99 samples from 33 surgically resected, stage I–III breast cancers; each cancer was biopsied in three distinct areas. Homologous recombination repair (HR) deficiency was defined as either high HRD score (≥42) or tumor BRCA mutation.

Results: Eighty-one biopsies from 32 cancers were analyzed. Tumor BRCA status was available for all samples, HRD scores for 70, and *BRCA1* methylation values for 76 samples. The *BRCA1/2* mutation and promoter methylation status and HR category showed perfect concordance across all biopsies from the same cancer. All tumors with *BRCA1/2* mutations or promoter methylation had high HRD scores, as did 17% (4/24) of the *BRCA1/2* wild-type and nonmethylated tumors. The HRD scores were also highly consistent between different biopsies from the same tumor with an intraclass correlation coefficient of 0.977, indicating that only 2.3% of the variance is attributed to within-tumor biopsy-to-biopsy variation.

Conclusions: These results indicate that within-tumor spatial heterogeneity for HRD metrics and the technical noise in the assay are small and do not influence HRD scores and HR status. *Clin Cancer Res; 23(5); 1193–9. ©2016 AACR.*

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Introduction

Many sporadic breast cancers have impairment in homologous DNA recombination repair due to somatic mutations and expression changes in key genes that mediate this process. Homologous recombination repair together with non-homologous end joining are the two principal ways to repair DNA double-strand breaks. This process can be conservative, involving the BRCA1 and BRCA2 protein in replacing a damaged DNA sequence with an identical sequence using the sister chromatid, or nonconservative and potentially mutagenic (single-strand annealing; refs. 1, 2). There is extensive preclinical evidence to suggest that cancers with homologous recombination deficiency (HRD) are particularly sensitive to DNA-damaging agents, including platinum compounds and PARP inhibitors. Several clinical trials demonstrated that platinum drugs and PARP inhibitors have increased activity in germline *BRCA1/2*-deficient tumors (3–5). However, some sporadic tumors (i.e., BRCA germline wild-type) also respond to these drugs, and up to 25% of these tumors show signs of HRD (6, 7).

It has been shown that a BRCA-deficient phenotype can be caused either by deleterious germline or somatic mutations in the BRCA genes or by methylation of the *BRCA1* promoter leading to low mRNA expression (8). Somatic or germline *BRCA1/2* mutations are observed in 10% to 40% of triple-negative breast cancers (TNBC) in patient populations known to be enriched for *BRCA1/2* mutations (9–13). Methylation of the *BRCA1* promoter is seen in around 15% to 25% in TNBC compared with <2% in all other breast cancer subtypes (8). In contrast, methylation of the *BRCA2* promoter is very rare in breast cancer (8). In addition to the BRCA genes, there are also a large number of other genes that are involved with HR and many of these are mutated, or their expression altered, in cancers. Whether these alterations result in similar increased sensitivity to DNA-damaging agents as the germline BRCA mutations is yet to be tested in the clinic.

Recently, a tumor DNA–based 3-biomarker HRD assay was developed that measures three distinct features of HR deficiency (14). These include a whole-genome loss of heterozygosity (LOH) profile score (15), a telomeric allelic imbalance score (TAI; ref. 16), and a large-scale state transitions score (LST; ref. 17). All three scores are highly correlated with germline defects in *BRCA1/2* and

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi: 10.1158/1078-0432.CCR-16-0889

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Translational Relevance

Several studies suggested spatial genomic heterogeneity within tumors, which might influence biomarker results from a single biopsy of a large tumor. Homologous recombination deficiency (HRD) is currently tested in clinical trials as a response marker for DNA-damaging agents and PARP inhibitors. We examined the intratumor variability of *BRCA1/2* mutation, *BRCA1* promoter methylation status, and the overall HRD score in small samples from different areas of the same cancer. We observed highly concordant HRD scores across different regions of the same cancer suggesting that HRD, when present, is a homogeneous feature of primary breast cancers. This feature makes it an attractive biomarker with low susceptibility to sampling error and also supports its value as a potential therapeutic target because the defect appears uniformly present and may confer cancer-wide sensitivity to drugs that exploit HR deficiency.

each, independently and in combination were shown to be associated with sensitivity to platinum agents (14–17). The 3-biomarker HRD assay is now being evaluated in clinical studies that examine its ability to identify patients who are particularly sensitive to DNA-damaging agents.

Several studies that examined the mutation landscape of different regions of the same tumor using deep sequencing showed differences in observed mutations depending on where the tumor was sampled (18–20). These findings suggest that spatial genomic heterogeneity exists within a single tumor and this might influence genomic biomarker results obtained from single biopsies. The same cancer may be called "positive" or "negative" for a particular genomic alteration depending on the biopsy site due to "sampling error" that represents the sum of biopsy-to-biopsy biological variation and inherent technical noise in the method including subtle differences in tissue composition between different biopsies. The goal of this study was to assess the reproducibility of HRD scores in small biopsy samples from different areas of the same primary breast cancers.

Materials and Methods

Breast tumor samples

Thirty-two patients were included in a prospective tissue sampling study to assess within tumor molecular heterogeneity using different platforms. This article reports on the HRD assay. Patients with newly diagnosed, chemo-naïve, invasive breast cancers measuring 1.5 cm or greater were recruited between January 9, 2012 and November 13, 2013 at the Yale Cancer Center. Cancer tissue samples were obtained with a punch biopsy tool from three different areas of the same primary tumor at least 1 cm apart by a pathologist or designated assistant after diagnostic gross examination had been completed. The samples were formalin fixed and paraffin embedded for DNA extraction by Myriad Genetics. This study was approved by the Yale Cancer Center Human Investigations Committee and all patients signed informed consent.

DNA extraction and HRD assay

The Promega Maxwell 16 FFPE Plus LEV DNA Purification Kit (cat. no. AS1135) was used to extract DNA from FFPE blocks and the HRD assay was performed by Myriad Genetics using next-generation sequencing (NGS). A custom capture panel targeting 54,091 different SNP loci and the coding regions of *BRCA1* and *BRCA2* was used. A detailed description of the panel design and development, and assay process is provided in Timms and colleagues (8). Sequencing was performed on an Illumina HiSeq 2500 according to the manufacturer's protocols (Illumina). To calculate the HRD score, reads covering SNP positions were used to generate allele imbalance profiles. A hidden Markov model was used to define regions and the corresponding break points within allele imbalance profiles. The TAI score was defined as the number of regions with allelic imbalance that extend to one of the subtelomeres but do not cross the centromere (16). The LST score is the number of break points between regions longer than 10 Mb after filtering out regions shorter than 3 Mb (17). Allele-specific copy number (ASCN) for each region was determined by an algorithm similar to that described in Popova and colleagues (17). ASCN was used to calculate the LOH score, which was defined as the number of subchromosomal LOH regions longer than 15 Mb (15). The HRD score is the unweighted sum of the TAI, LOH, and LST scores. Each sample was also examined for tumor mutations in *BRCA1* and *BRCA2* and for promoter methylation of *BRCA1*. Descriptions of the sequence alignment and mutation detection methods used to define *BRCA1/2* mutation status are provided in Timms and colleagues (8). The percentage of *BRCA1* promoter methylation was determined by quantitative NGS assay that assesses methylation at 10 distinct CpG sites within the CpG island. The quantitation reflects the frequency of methylated reads versus unmethylated reads. A site was defined as methylated if the proportion of methylated reads is $\geq$10%. Samples with either zero or one methylated CpG site were considered not to be methylated. Samples with either nine or ten methylation CpG sites were considered to be methylated. All other reads were ignored because their methylation status is ambiguous. BRCA deficiency was defined as either deleterious mutation or methylation.

Samples were assigned into high ($\geq$42) or low (<42) HRD score based on a threshold that was previously established in a separate discovery sample set and represents the 5th percentile of HRD scores in tumors with BRCA deficiency (14). Homologous DNA recombination deficiency was defined as either a high HRD score or mutation in *BRCA1* or *BRCA2* genes.

Statistical analysis

We used linear mixed effects regression modeling to estimate the variance in HRD scores across tumors and between replicate samples from the same tumor. We used the Wilcoxon rank test to compare the difference of pairwise HRD scores between replicate samples from the same tumor and samples from different tumors. To evaluate whether the within tumor variance depends on HRD status, we used a heteroscedastic model that allows to compute differences between biopsy variances for the HRD-low and HRD-high tumors. The intraclass correlation coefficient was calculated as the ratio of the between sample variance over total variance. All computations were performed in R v3.1.2 (R Core Team (2014, http://www.R-project.org/) using the nlme library (21).

Results

Table 1 lists patient and clinical pathologic characteristics for all patients included in the study. The majority of tumors were high grade, hormone receptor positive, HER2 negative, and clinically

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Table 1. Patient and clinical pathologic characteristics

Characteristic	Number of patients	%
Age at tissue sampling, y		
Median	62	
Range	27–90	
Tumor		
Nuclear grade		
1	0	0%
2	13	39.4%
3	20	60.6%
Hormone receptor status		
ER^+/PR^+	18	54.6%
ER^+/PR^-	5	15.1%
ER^-/PR^+	1	3.0%
ER^-/PR^-	9	27.3%
HER2 status		
Negative	28	84.8%
Positive	5	15.2%
Clinical stage		
IA	5	15.2%
IIA	20	60.6%
IIB	7	21.2%
IIIC	1	3.0%

Abbreviation: y, years.

stage IIA. Figure 1 is a consort diagram that shows sample disposition. The use of the specimens for the analyses is displayed in Table 2. Of the 99 biopsy specimens from 33 tumors (33 × 3), 15 contained either no tissue or <5% tumor cellularity and were not extracted. DNA extraction was performed on the remaining 84 FFPE blocks, 81 yielded sufficient DNA for molecular analysis and generated 81 tumor BRCA calls (32 patients), 76 *BRCA1* promoter methylation values (31 patients), and 70 HRD scores (31 patients, including 3 biopsies from 16, two biopsies from 7, and 1 biopsy from 8 patients).

Twelve of 81 (14.8%) tumor specimens had deleterious tumor *BRCA1* ($n = 3$) or *BRCA2* ($n = 9$) mutations corresponding to 4 of 32 (12%) patients who had BRCA sequencing data available (Fig. 2). The one tumor with the *BRCA1* mutation was triple negative, and all three *BRCA2* mutant tumors were hormone receptor positive and HER2 negative. Germline BRCA results were not available for any of these cases. *BRCA1/2* mutation status was consistent across all samples from the same cancer resulting in perfect concordance. All tumor *BRCA1*- or *BRCA2*-mutant cases had loss of heterozygosity in the unaffected allele. We also detected 2 variants of unknown significance in the *BRCA1* gene. In one case, *BRCA1* c.548-16G>A (IVS8-16G>A; chr17:41249322), there was no other BRCA mutation or *BRCA1* promoter methylation, and the overall HRD score was low. Of note, the variant was detected in all 3 samples from that case. The other variant, *BRCA1* c.358G>C (p.Asp120His; chr17:41256222) was also present in all samples from the case. This cancer had a deleterious *BRCA2* mutation with loss of heterozygosity in the other allele. The overall HRD score for this case was also high.

Six of 76 (8%) samples had methylation of the *BRCA1* promoter corresponding to 2 of 31 (6.5%) patients who had methylation data available (Fig. 2). One tumor was TNBC; the other hormone receptor positive, HER2 negative. Similar to BRCA mutations, promoter methylation (or the lack of it) was uniformly detected in all small samples from the same tumor, yielding a perfect concordance for multiple biopsies from the same case.

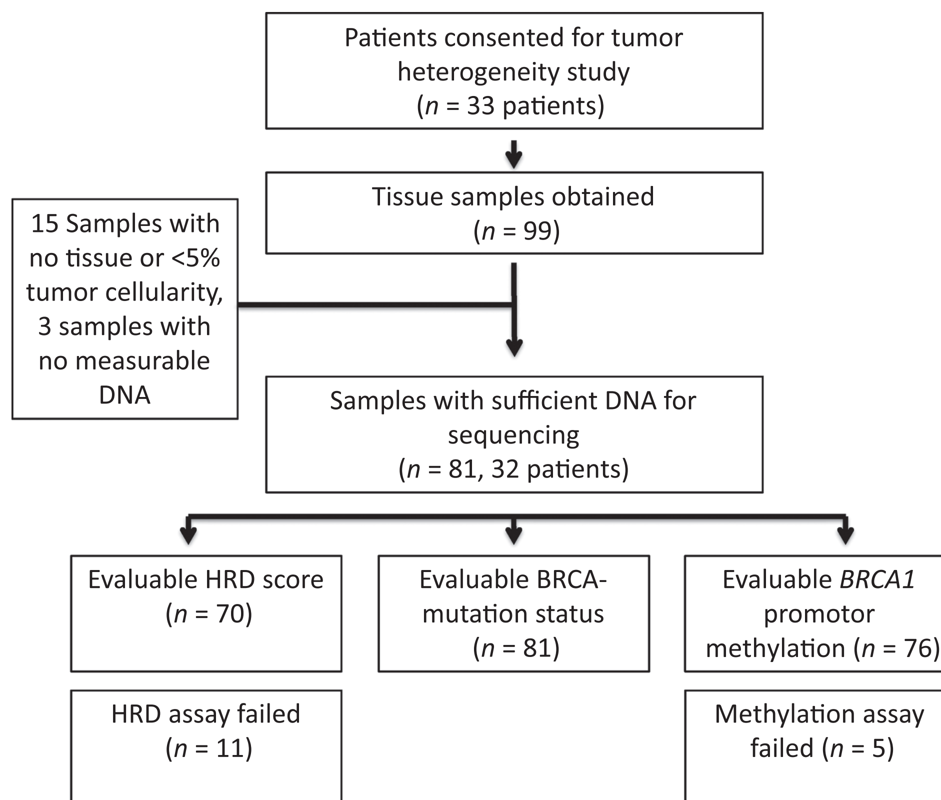

Figure 1.
Consort diagram. This diagram shows the disposition of the samples from 33 patients.

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Table 2. Use of specimens for analyses

	Samples	Patients	Triplets	Duplicates	Singles
Total tumor samples	99	33	33	—	—
Sufficient DNA for sequencing	84% (81/96)	32	21	7	4
Evaluable tumor *BRCA1/2* mutation data	100% (81/81)	32	21	7	4
BRCA1	100% (81/81)	32	21	7	4
BRCA2	100% (81/81)	32	21	7	4
Evaluable HRD scores	86% (70/81)	31	16	7	8
High HRD score	36% (25/70)	10	38% (6/16)	43% (3/7)	12% (1/8)
BRCA1 mutation	4% (3/81)	1	5% (1/21)	0% (0/7)	0% (0/4)
Evaluable *BRCA1* promotor methylation	94% (76/81)	31	19	7	5
BRCA1 promotor methylation	8% (6/76)	2	11% (2/19)	0	0
BRCA2 mutation	11% (9/81)	3	14% (3/21)	0% (0/7)	0% (0/4)

Twenty-five of the 70 samples (36%) had high HRD scores corresponding to 10 of 31 patients (32%) who had HRD score available. All tumors with BRCA deleterious mutations or *BRCA1* promoter methylation had high HRD scores (Fig. 2 and 3), as did 17% (4/24) of the *BRCA1/2* wild-type, *BRCA1* unmethylated tumors (Fig. 2 and 3). HRD scores and component scores (LST, TAI, LOH) for all 70 samples are provided in Supplementary Table S1. The standard deviations of HRD scores in multiple samples from the same tumor were small and HRD scores were highly consistent between independent biopsies of the same tumor (Fig. 3). This was true for all matched biopsies, including those with significant differences in genomic rearrangements (Supplementary Fig. S1). The pairwise differences of HRD scores were significantly less in samples from the same tumor (median = 2) compared with difference in HRD scores between samples from different cancers (median = 22), Wilcoxon rank test $P < 2.2 \times 10^{-16}$ (Fig. 4A). No tumor harbored greater intratumor difference in HRD score than the median level of the intertumor difference (Supplementary Fig. S2A). The SD of the HRD scores within replicate samples from the same cancer over all samples, estimated from the mixed effects analysis, was 2.67, and no significant heteroscedacity was detected between the HRD-low and HRD-high cases (likelihood ratio test comparing homoscedastic and heteroscedastic model $P = 0.2$). The intratumor difference of HRD scores was significantly less than the intertumor difference in cancers with both high ($P = 6.1 \times 10^{-9}$, Fig. 4B) and low ($P = 4.4 \times 10^{-14}$, Fig. 4C) HRD scores. The median of intertumor HRD score differences was 11 in cancers in the low and 14 in cancers in the high HRD categories, respectively (Supplementary Fig. S2B and S2C).

The HRD status (i.e., deficient vs. not) was the same in all of the small samples from different areas of the same cancer, in all 32 tumors (Fig. 2). The intraclass correlation coefficient for HRD score was 0.977, indicating that only 2.3% of the total observed variance is attributed to within-tumor biopsy-to-biopsy variation.

Discussion

HRD, including *BRCA1/2* aberrations, is currently tested in clinical trials as a potential molecular marker of response to

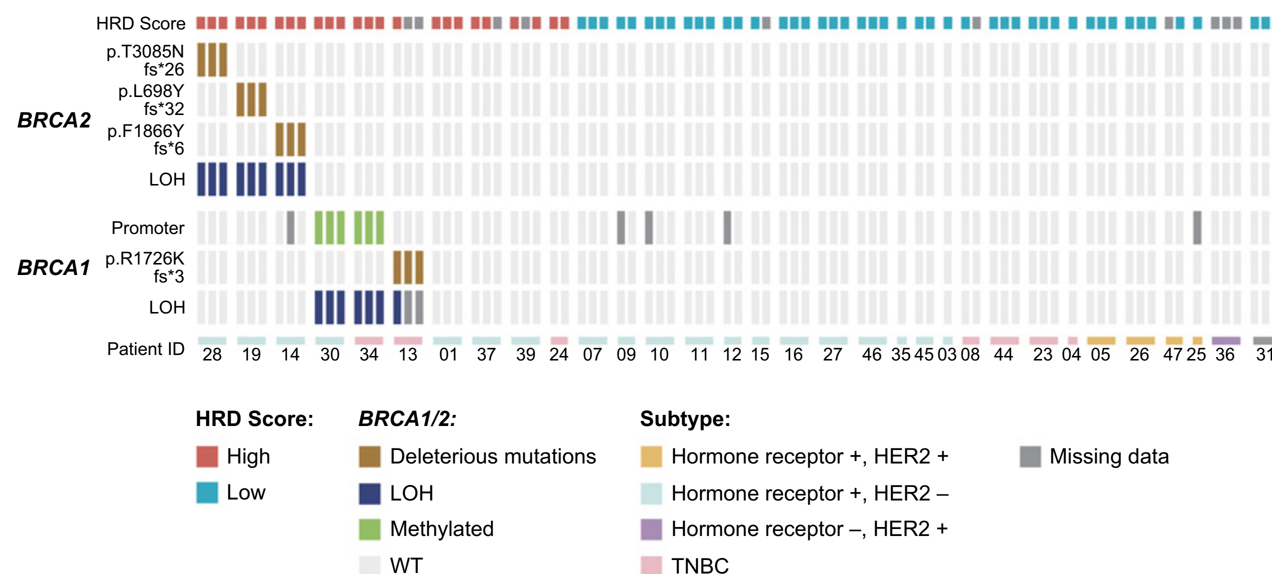

Figure 2.
Genomic abnormalities of *BRCA1/2* and HRD scores in 81 samples corresponding to 32 breast cancer patients. Each sample was assigned into high (≥42) or low (<42) HRD score, and multiple samples from the same patient were grouped together. Deleterious mutations, LOH events, and promoter methylation are shown for *BRCA1* and *BRCA2* separately.

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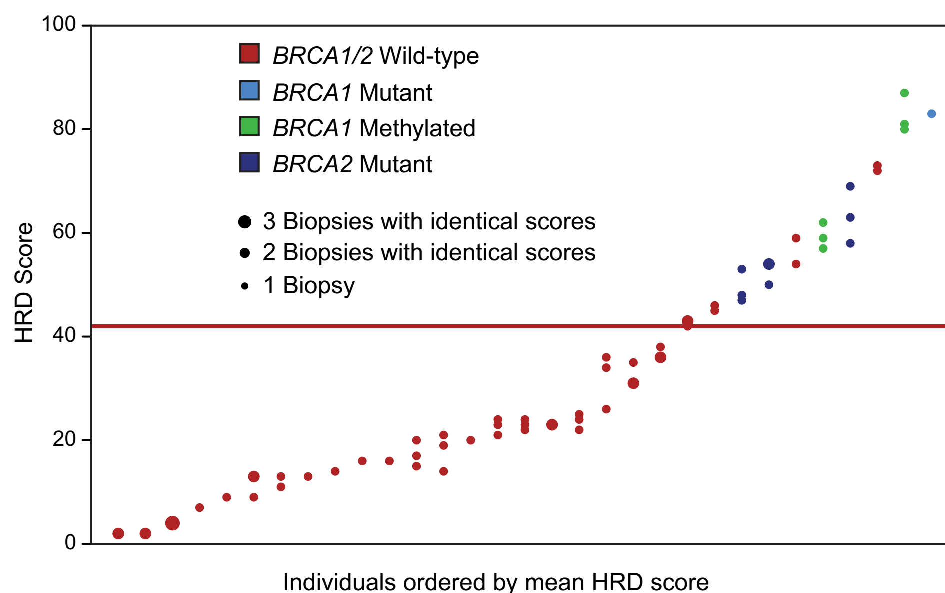

Figure 3.
Scatter plot of HRD scores from multiple biopsies from the same tumor. Points are vertically aligned for each individual patient and ordered from left to right by increasing mean score. Dots are colored by *BRCA1/2* mutation status (red, *BRCA1/2* wild-type; blue, *BRCA1* mutant; green, *BRCA1* methylated; black, *BRCA2* mutant). The size of the dots indicates the number of biopsies with identical scores (3, 2, or 1).

DNA-damaging agents and PARP inhibitors. HRD status is often assessed in core-needle biopsy samples of the cancer, or in a few tumor tissue sections from a tumor resection specimen that represent only a small fraction of a larger tumor mass. The purpose of this study was to examine the consistency of HRD measures and the 3-biomarker HRD assay score in small samples from different areas of the same cancer. If the results were frequently discordant this could reflect low technical reproducibility of the assay, high within tumor heterogeneity for HRD metrics, or a combination of these, all limiting the clinical utility of the test.

All components of the HRD score, including *BRCA1/2* mutation and *BRCA1* promoter methylation as well as the HRD score itself, were highly consistent between all samples from different areas of the same cancer. High HRD scores ($\geq$42) were observed in all cancers with *BRCA1/2*-deficient mutations and *BRCA1* promotor methylation and also in several BRCA wild-type cancers.

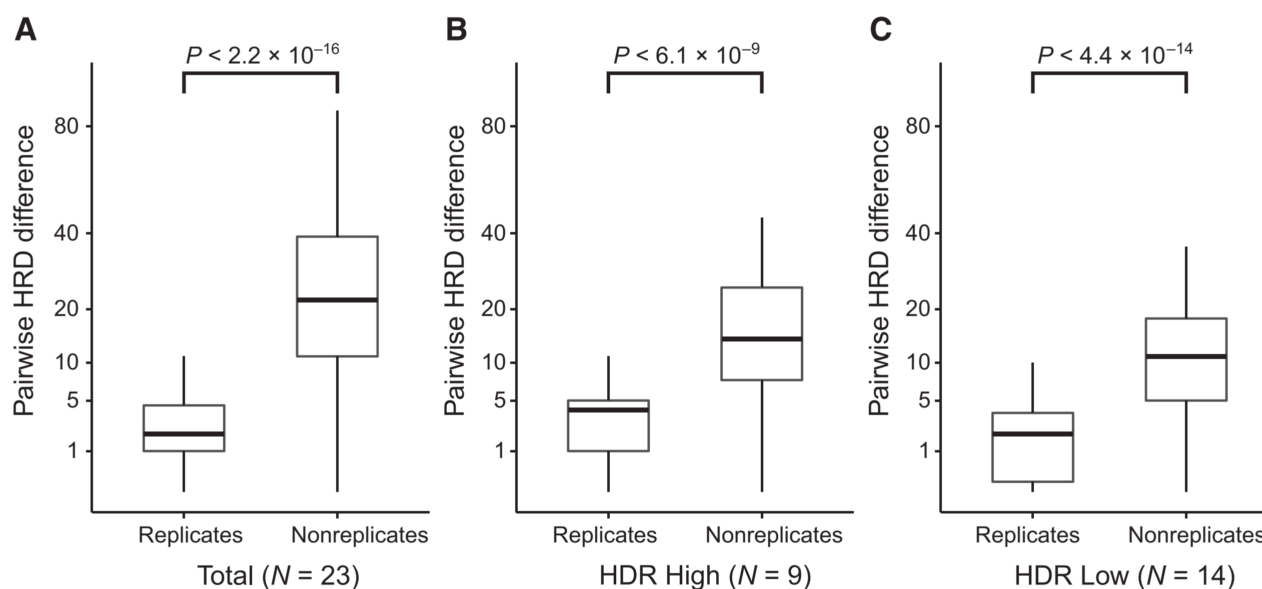

Figure 4.
Concordance of the HRD scores. We computed the pair-wise HRD score between any two samples and considered samples as replicates if they were from the same patient and nonreplicates if they were from different patients. **A,** Distribution of pair-wise HRD score difference between replicates was significantly lower than those between nonreplicates by the Wilcoxon rank test ($P < 2.2\times10^{-16}$) for all samples. **B,** Distribution of pair-wise HRD score difference between replicates was significantly lower than those between nonreplicates by the Wilcoxon rank test ($P < 6.1\times10^{-9}$) for samples of high HRD scores. **C,** Distribution of pair-wise HRD score difference between replicates was significantly lower than those between non-replicates by the Wilcoxon rank test ($P < 4.4\times10^{-14}$) for samples of low HRD scores.

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We observed high HRD scores in both estrogen receptor–positive and triple-negative cancers. Overall, 12.5% had *BRCA1* or *2* mutations, an additional 6.4% had *BRCA1* promotor methylation and the remaining 13.1% had an unspecified defect in the homologous repair pathway that was detected on the basis of the 3-biomarker HRD assay alone. These frequencies are within previously reported ranges of these measures (8, 22). The most likely explanation for high HRD scores in BRCA-normal cancers is that these have defects in other known (e.g., PALB2, RAD51C, and RAD51D) or yet to be identified genes involved in homologous recombination. It is therefore possible that the HRD score may have utility beyond BRCA deficiency by identifying more patients who may be highly sensitive to DNA-damaging agents or PARP inhibitors.

The highly concordant HRD scores and HR metrics across different regions of the same cancer indicate that defects in homologous recombination effect the entire primary tumor. Genomic alterations that are uniformly distributed in all cancers cells are often events that provide selective advantage (23). The absence of substantial within tumor heterogeneity makes the HRD assay an attractive biomarker with low susceptibility to sampling error. It also supports its value as a potential therapeutic target since the defect is uniformly present and may confer cancer-wide sensitivity to drugs that exploit HR deficiency.

In summary, we demonstrated high reproducibility of the HRD assay with low technical and intratumor variability. Our results indicate a uniform HR deficiency status across different regions of primary breast cancers.

Disclosure of Potential Conflicts of Interest

K.M. Timms, A. Gutin, and J.S. Lanchbury have ownership interests (including patents) in Myriad Genetics. L. Pustzai is a consultant/advisory board member for Biotheranostics. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-K. von Wahlde, K.M. Timms, T. Jiang, J. Reid, Al. Gutin, C. Hatzis, L. Pusztai
Writing, review, and/or revision of the manuscript: M.-K. von Wahlde, K.M. Timms, A. Chagpar, V.B. Wali, T. Jiang, V. Bossuyt, J.S. Lanchbury, C. Hatzis, E. Hofstatter, L. Pusztai
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Acknowledgments

We thank Krystal Brown for her assistance with article preparation. We also acknowledge funding from the Connecticut Breast Health Initiative.

Grant Support

L. Pusztai is supported by the Breast Cancer Research Foundation and M.K. von Wahlde is supported by a Research Fellowship from the German Research Foundation (WA 3514/2-1). A. Chagpar is supported by the American Society of Breast Surgeons Foundation.

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Received April 15, 2016; revised July 21, 2016; accepted August 4, 2016; published OnlineFirst September 6, 2016.

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