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Bi-allelic alterations in DNA repair genes underpin Homologous recombination DNA repair defects in breast cancer

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Abstract

Homologous recombination (HR) DNA repair deficient (HRD) breast cancers have been shown to be sensitive to DNA repair targeted therapies. Burgeoning evidence suggests that sporadic breast cancers, lacking germline *BRCA1*/*BRCA2* mutations, may also be HRD. We developed a functional *ex-vivo* RAD51-based test to identify HRD primary breast cancers. An integrated approach examining methylation, gene expression and whole-exome sequencing was employed to ascertain the etiology of HRD. Functional HRD breast cancers displayed genomic features of lack of competent HR, including large-scale state transitions and specific mutational signatures.

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Data and materials availability: Code used to compute LST, ntAI, HRD/LOH, and perform analysis of mutational signatures is available from the authors upon request. Sequencing data are in process of being submitted to dbGaP and will be available under accession to-be-determined.

Somatic and/or germline genetic alterations resulting in bi-allelic loss-of-function of HR genes underpinned functional HRD in 89% of cases, and were observed in only one of the 15 HR-proficient samples tested. These findings indicate the importance of a comprehensive genetic assessment of bi-allelic alterations in the HR pathway to deliver a precision medicine-based approach to select patients for therapies targeting tumor-specific DNA repair defects.

Keywords

BRCAness; HRD; RAD51; DNA repair; mutation

Introduction

Homologous recombination (HR) plays a critical role in the repair of double strand breaks (DSBs), replication-associated DNA damage, and inter-strand crosslinks.(1) Germline mutations affecting specific known HR repair genes result in an increased risk of breast cancer development.(2) For example, *BRCA1* and *BRCA2* germline mutations are present in approximately 5–7% of all breast cancers.(3) The protein products encoded by the BRCA1/2 genes are essential members of the HR pathway, assisting in the maintenance of genomic integrity. In the absence of HR, DSBs are repaired by more error-prone mechanisms, such as non-homologous end joining, leading to genomic instability and tumorigenesis. Cells with homologous recombination deficiency (HRD) have been shown to be exquisitely sensitive to platinum-based chemotherapy and poly(ADP-ribose) polymerase (PARP) inhibitors, which produce replication-associated DSBs. Therefore, HRD has been targeted in cancers with the aim of exploiting a tumor-specific deficiency in DNA repair.(4) This “synthetic lethal” approach has recently led to the approval of PARP inhibitors for *BRCA1/2*-associated ovarian cancers and the investigation of cisplatin and PARP inhibitors in *BRCA1/2*-associated breast cancers.(5–8)

There are burgeoning data suggesting that HRD is likely present in a subset of non-BRCA1/BRCA2-mutant sporadic breast cancers.(9) The etiology of HRD in sporadic breast cancer, however, still remains unclear and the identification of these tumors in the clinic remains challenging. HRD in cancer results in a distinctive pattern of genomic instability due to the deficiency in error-free DNA double strand break repair by HR.(10–12) Therefore, biomarkers based on genomic landscape ‘scars’ or ‘footprints’ (i.e. patterns of somatic genetic alterations assessed by large-scale state transitions (LST), telomeric regions with allelic imbalance (NtAI), or large segments with loss-of-heterozygosity (Myriad LOH/HRD)), which are commonly seen in *BRCA1/2* associated breast cancer due to HRD, have been proposed for the identification of sporadic breast cancers with HRD.(13–16) Although these genomic landscape biomarkers correlate well with *BRCA1/2* germline mutations, their clinical utility in breast cancer has been limited because of their modest positive predictive value.(17, 18) One potential explanation for these observations is that these genomic ‘scars’ may develop early during tumor evolution, but will continue to be detected even if the cancer cells have re-acquired competent HR at the time of therapeutic decision-making.(19, 20)

The DNA recombinase RAD51 forms a focus at DNA damage sites, which are visible by immunofluorescence microscopy, and mark sites of ongoing DNA repair. The recruitment of RAD51 to single strand DNA, catalyzes strand invasion, and is a crucial step in HR that is dependent on the functional integrity of the entire pathway.(1) Hence, the assessment of RAD51 has been proposed as a surrogate for competent HR DNA repair, however previous approaches require patients to receive systemic cytotoxic therapy within a few hours to days prior to the tumor biopsy for biomarker assessment.(21) To address the unmet need of a test that accurately assesses the functional status of HR at the time of diagnosis, we utilized a functional RAD51 assay to measure HR in prospectively accrued human breast cancer specimens. After benchmarking this assay on the basis of the clinicopathologic and genomics features of the tumors, we sought to define the underlying etiology of HRD in breast cancers employing a multi-faceted genomic approach (Fig. 1).(22)

MATERIALS AND METHODS

Patients

We obtained fresh and flash frozen tumor specimens from 56 breast cancer patients diagnosed between August 2010 and April 2012. (Supplementary Table 1). This study was approved by the Institutional Review Board, and informed consent was obtained from all patients prior to enrollment. Cases were anonymized prior to functional and genetic analyses. Details of inclusion/exclusion criteria are described in the Supplementary Methods.

Ex Vivo treatment and DNA repair protein foci assay of homologous recombination

Following excision and without delay, the lumpectomy or mastectomy specimen was grossly assessed by a breast cancer pathologist and a fraction of the tumor was set-aside in chilled complete cell culture medium. A cell suspension was created and divided equally, with one half being irradiated with 10Gy, while the other half was mock-treated (i.e. not irradiated). The samples were then incubated in for 4 hrs, after which they were mounted on glass slides. Cell nuclei were analyzed for subnuclear foci formation of RAD51 in both the irradiated and mock-treated (i.e. non-irradiated) states as a functional readout of HR. IR-induced γH2AX foci formation was analyzed to assess the quality of the preparation and cell viability at the time of DNA damage and fixation. BRCA1 foci formation was also assessed to facilitate the localization of potential defects in the HR pathway. For example, lack of both BRCA1 and RAD51 IR-induced foci formation would suggest a defect upstream of BRCA1 in the HR pathway. By contrast, IR-induced BRCA1 foci formation in the absence of RAD51 foci formation would suggest the HR defect is due to a deficiency downstream of BRCA1. At least 200 nuclei were counted for both the irradiated and non-irradiated conditions of a given case. A nucleus was scored as positive if it contained >5 foci, as previously described.(22) P-values were calculated using a two-sided test of proportions with a Z-test, and $P<0.05$ was considered statistically significant.

Immunohistochemistry

Immunohistochemical (IHC) analysis was performed on the matching formalin-fixed, paraffin-embedded tissue sections of the breast cancers included in this study using

antibodies against PCNA and Ki67 using standard procedures and validated controls (Supplementary Methods).

Nucleic acid extractions

DNA and RNA were extracted from representative flash frozen tumor sections using the DNeasy Blood & Tissue kit (Qiagen) and TRIzol (Life Technologies), respectively (Supplementary Methods).

BRCA1 promoter methylation

100ng genomic DNA from each breast cancer was bisulfite converted using the EpiTect Plus Bisulfite Kit (Qiagen). Purified converted DNA was subjected to methylation-specific PCR (MSPCR) using the EpiTect MSP Kit (Qiagen, Supplementary Methods).

Whole-exome sequencing and copy number analysis

DNA extracted from snap-frozen tumors and germline were subjected to whole exome capture using the SureSelect Human All Exon v4 (Agilent) capture system and to massively parallel sequencing on an Illumina HiSeq 2000 following validated protocols. Whole-exome sequencing analysis was performed as described in Weinreb *et al.* with modifications (Supplementary Methods). Somatic single nucleotide variants (SNVs) were identified using MuTect and small insertions and deletions (indels) were identified using VarScan2 and Strelka. For copy number analysis and detection of loss of heterozygosity (LOH), OncoSNP-Seq (v2.0) was employed. Prior to analysis, two authors (S.N.P, N.R.) curated a list of 95 genes that are direct or indirect effectors or regulators of HR using the literature and author experience.(23, 24) Comparison of the number of cases with the complete loss of both alleles of at least one HR gene according to functional RAD51 foci formation status was performed using Fisher's exact test.

Analysis of genomic 'scars'

Large-scale state transitions (LST), telomeric regions with allelic imbalance (NtAI), or large segments with loss of heterozygosity (Myriad LOH/HRD) scores were derived from whole-exome sequencing data by first extracting heterozygous SNPs and allele specific copy number estimates from the exome data. LST, ntAI, and HRD scores from allele specific segmented data were determined following methods outlined in the initial publications and described in detail in the Supplementary Methods.(13–15)

Analysis of mutational signatures

To measure the mutational context of all somatic synonymous and non-synonymous SNVs present in a given sample, the 5′ and 3′ sequence context of each mutation was extracted from the GRCh37. Mutational signatures were defined using whole-exome sequencing data as described in Supplementary Methods.

Results

Functional Analysis of RAD51 Foci Formation to Define HR DNA Repair Defects

HRD was evaluated using a quantification of RAD51 foci in cancer cells subjected to *ex-vivo* ionizing radiation (IR), which has previously been shown to be a robust readout of the integrity of HR *in-vitro*.(22) We obtained tumor specimens from 56 consecutive patients with breast cancers prospectively (Table 1). Briefly, immediately after surgical resection, we generated single cell suspensions from each tumor. For each patient, half of these suspensions were irradiated with 10 Gy, while the other half was mock-treated (i.e. un-irradiated). Cell nuclei were analyzed for the formation of RAD51 foci in both irradiated and un-irradiated cells using confocal microscopy. In addition, we assessed γH2AX and BRCA1 foci formation, as described above. To ascertain that RAD51 deficiency was not due to cellular quiescence, we used immunohistochemical analysis of the proliferation marker Ki67 (Supplementary Fig. 1a–b). As HR is limited to the S/G2 phases of the cell cycle and an absence of RAD51 induction denotes HRD, we only considered cases for further analysis if they showed sufficient levels of Ki67 staining (proficient >5%; deficient >20%; Supplementary Fig. 2). Forty-nine tumors had sufficient levels of proliferation, as defined by Ki67, for subsequent analysis. By assessing the induction of RAD51 foci formation in irradiated vs mock-irradiated cells, we observed that 78% (38/49) of the tumors displayed a significant increase in the number of cells with RAD51 foci following IR (Figs. 2a,c,e), a phenotype we classified as “RAD51 proficient”. In addition, 22% (11/49) of tumors lacked a significant increase in RAD51 foci following IR (Figs. 2b,d,e). We classified these tumors as “RAD51 deficient”.

The relative fold-increase in RAD51 recruitment following IR displayed a clear bi-modal distribution in the breast cancers analyzed (Fig. 2e). All 38 RAD51 proficient tumors also induced BRCA1 foci following IR. In 7 of the 11 tumors classified as RAD51 deficient, there was also no induction of BRCA1, whereas 4 RAD51 deficient tumors exhibited a 2 to 5 fold increase in cells with BRCA1 foci following IR. Notwithstanding these 4 cases, induction of RAD51 foci was linearly related to induction of BRCA1 foci ($r = 0.91$, $p < 0.001$, Supplementary Fig 1c). RAD51 deficiency (i.e. functional HRD) was detected in 11 breast cancers and observed in all clinical subtypes. A numerically but not statistically significant higher prevalence of functional HRD, however, was documented in triple-negative breast cancers (42%, Fig. 2f). No association between HRD and other clinico-pathologic features was observed (Table 2).

Relationship between functional HR assays and genomic ‘scars’

We next sought to define whether breast cancers with functional HRD, as defined by the *ex-vivo* RAD51 assay, would display genomic ‘scars’ or mutational signatures consistent with HRD.(13–16, 25, 26) A subset of 24 tumors from which sufficient DNA was available, including nine RAD51-defective tumors and 15 RAD51-foci-positive controls (Supplementary Tables 1 and 2), was subjected to whole-exome sequencing. Consistent with our hypothesis, tumors with functional HRD (i.e. RAD51-deficient) had significantly higher number of *BRCA1/2*-like genomic ‘scars’ than HR-proficient breast cancers. The LST, ntAI, LOH/HRD scores, and the number of insertions and deletions (indels) were significantly

higher in tumors with functional HRD (Wilcoxon rank-sum test p=0.002, p=0.009, p=0.048 and p=0.044, respectively; Fig. 3a–c). The positive predictive value, negative predictive value, and accuracy of LST using a cut-off of 15 (as per initial report (13)) to determine RAD51 functional status were 59%, 90%, and 82%, respectively. In addition, using a validated approach to classify cancers into the 21 mutational signatures that shape the genomes of human cancers (25), we observed that the *BRCA1/2* mutational signature (signature 3) was present in 4/9 (44%) RAD51-deficient breast cancers but in none of the 15 RAD51-proficient cases (p=0.02, Fisher's exact test, Fig. 5), suggesting that this signature may only identify a subset of breast cancers with HRD (i.e. three of five tumors with *BRCA1* and *BRCA2* pathogenic mutations did not display the *BRCA1/2* mutational signature). Taken together, we demonstrate that HRD breast cancers as defined by a functional RAD51 foci assay display the expected cardinal genomic features of breast cancers lacking competent HR DNA repair (e.g. those of BRCA1/2 hereditary breast cancers).

Integrated Genetic Analysis HR Deficient and Proficient Tumors

We next sought to identify the etiology of functional HRD. mRNA levels of a panel of HR genes, including *BRCA1*, *BRCA2*, *RAD51*, *RNF168*, and *RAP80* and *FAM175*, were tested in HRD and HR DNA repair competent cases using NanoString (Fig. 4). The expression levels of the HR genes were found not to be associated with HRD. Similarly, *BRCA1* gene promoter methylation was also not associated with functional HRD status in tumors analyzed although just two BRCA1 methylated cases were identified in this cohort.

Given that alterations in multiple HR genes in addition to BRCA1*/2* have been associated with either predisposition to breast or ovarian cancer or response to DNA damaging chemotherapy,(2, 27) we posited that functional HRD may be underpinned by genetic alterations that target distinct components of the HR pathway in sporadic breast cancers. Importantly, there is evidence to suggest that for most HR genes, bi-allelic loss is essential for cancer cells to be HR DNA repair deficient.(9, 28–30) Whole-exome sequencing analysis revealed that bi-allelic germ-line and/or somatic genetic alterations affecting 95 previously-reported HR DNA repair pathway genes (Supplementary Table 3) accounted for the functional HRD observed in 8/9 (89%) cases analyzed (Fig 5 and Supplementary Table 4–6). (23, 24) For instance, 4/9 patients with functional HRD harbored alterations in *BRCA2* (Fig. 5), all of which likely resulted in a complete loss of *BRCA2* (germline frameshift mutation with a somatic LOH (Case SP15), somatic frameshift mutation with LOH (Case SP28), a somatic exon 3 duplication with LOH (Case SP5), and a somatic homozygous deletion (Case SP17). Consistent with its role upstream of BRCA2 in the HR pathway, IR-induced BRCA1 recruitment into DNA repair foci was preserved in these four tumors. Four additional HRD cases had bi-allelic alterations of bona fide HR genes, including one case with a *CHEK2* somatic homozygous deletion (Case SP6). Loss of *CHEK2* diminishes RAD51 recruitment to the sites of DNA damage following IR (unpublished observation). (31, 32) The two cases with somatic homozygous deletions of either *BRCA2* (Case SP17 or *CHEK2* (Case SP6) had negligible mRNA expression levels of the corresponding gene (Supplementary Fig. 3), providing additional evidence of the functional consequence of the homozygous deletions detected. Two additional HRD cases showed non-synonymous

somatic mutations and LOH in *FAAP100* (Cases SP16, SP26), a Fanconi Anemia associated protein. Integrity of the Fanconi anemia pathway is required for RAD51 recruitment and HRD results when this pathway is inactivated.(33) Another case had a mutation and LOH in *TP53BP1*, which may result in a switch from repair of double strand breaks with fidelity by HR, to a reliance on RAD52-mediated mutagenic single-strand annealing.(34) Consistently, this tumor exhibited the highest number of indels, suggesting greater genomic instability. Case SP6, in addition to a *CHEK2* homozygous deletion, also harbored a homozygous deletion in *BABAM1* (MERIT40 or NBA1), a member of the BRCA1-A complex known to affect BRCA1 and RAD51 recruitment.(35) The only RAD51 foci formation proficient case displaying a bi-allelic inactivation of an HR gene was case SP20. This tumor despite harboring a germline frameshift mutation in *BRCA1* coupled with somatic LOH of the wild-type allele, was found to be proficient for induced RAD51 foci and BRCA foci and did not have an elevated LST score or a BRCA mutational signature. In addition, this case did not display evidence of intra-genic deletions or reversion mutations in the tumor, nor did it have low expression of 53BP1, suggesting there might be additional mechanisms that can restore HR function in these tumors.(36–38) In total, 8/9 of RAD51-deficient cancers harbored a bi-allelic inactivation of at least one HR gene compared to 1/15 of RAD51-proficient cancers ($p<0.001$, Fisher's exact test), suggesting these eight cases likely had a genetic etiology for functional HRD. The sole case that was RAD51 deficient but did not contain a bi-allelic inactivation affecting one of the 95 HR DNA repair-related genes, also failed to significantly induce BRCA1 foci following IR but did not have *BRCA1* promoter methylation nor any obvious difference in gene expression of BRCA1 or the other HR genes, as assessed by nanostring. The lack of a large number of LSTs and indels, in addition to the absence of the mutational signature 3 suggest a genetic alteration not surveyed by whole-exome sequencing (e.g. somatic genetic alterations affecting non-protein coding regulatory elements or genetic rearrangements) or an epigenetic alteration may have led to deficiency in this case. Of note, single-allelic alterations in HR genes occurred in 12 cases and were associated with RAD51-deficiency, albeit less strongly than bi-allelic inactivation. ($p=0.01$; Fisher's exact test; Supplementary Fig. 4).

The nine cases with bi-allelic inactivation of HR DNA repair genes, including the *BRCA1* germline mutated but RAD51-proficient case, were found to have a significant association with higher LST scores ($p=0.001$, Wilcoxon rank-sum test, Fig. 3d). To determine whether this association would be generalizable, we performed an analysis of breast cancer samples from The Cancer Genome Atlas (TCGA) study (Methods).(39) In the TCGA dataset, breast cancers with a bi-allelic genetic alteration in the HR pathway gene panel also displayed significantly higher LST scores than those that did not ($p < 0.001$, Wilcoxon rank-sum test, Supplementary Fig. 5).

Taken together, our findings demonstrate that in 8 of 9 breast cancers displaying functional HRD, the lack of competent HR DNA repair was likely caused by bi-allelic genetic inactivation of a bona fide HR-related gene. Although we included *TP53BP1* in our gene panel of HR regulators and effectors, *a priori*, we acknowledge that mutations in this gene may promote HR, especially in a *BRCA1* mutant background (importantly this is not the case here). Further, emerging evidence, suggests that TP53BP1 plays a critical role in supporting the accumulation of RAD51 at IR-induced DNA double strand breaks. Rather

than suppressing HR in a *BRCA1* wild-type background, loss of 53BP1 may trigger a hyper-resection phenotype, leading to replacement of RAD51 by RAD52 and redirecting repair from HR to more mutagenic single-strand annealing.(34) Nevertheless, excluding this case (i.e. only 7 of 9 cases with bona-fide bi-allelic HR genes) does not significantly alter our findings. Bi-allelic alterations are still significantly associated with RAD51 deficiency and correlate with LST ($p < 0.001$ and $p < 0.01$; Fisher's exact test and Wilcoxon-rank sum test, respectively).

Discussion

Here, we developed and validated an ex-vivo functional assay for the identification of HRD breast cancers. This assay revealed that over 20% of the breast cancers analyzed were found to have a functional deficiency in the HR pathway. This RAD51 foci-induction assay is the only HRD classifier to display a bimodal distribution, suggesting that there is a biologically driven categorization of breast cancers by status of the HR pathway. Breast cancers classified as functionally HRD displayed the cardinal genomic features reported to be present in tumors lacking competent HR, including high LST scores and the BRCA mutational signature (i.e. signature 3). Although HRD was most frequently observed in triple-negative breast cancers, this functional deficiency was also present in ER-positive and/or HER2-positive disease. An integrative genomic analysis of cases with and without HRD revealed that the likeliest etiology for HRD in the vast majority of cases is *bi-allelic inactivation* of *bona fide* HR genes, and that *BRCA1* gene promoter methylation and transcriptomic changes in HR genes were not associated with functional HRD. These observations demonstrate that HRD is predominantly caused by genetic events during tumorigenesis and tumor evolution, and that this phenomenon likely constitutes a convergent phenotype in breast cancers. (9, 40)

Germline variants in HR genes besides *BRCA1/BRCA2* are associated with breast cancer predisposition, and underlie the importance of assessing the genotype of the entire pathway. (2, 41) Genetic alterations affecting HR pathway-related genes have been linked to response to HR-targeted therapies in multiple other cancers.(27, 42, 43) In ovarian cancer, somatic and germline assessment of a panel of 13 HR genes was significantly associated with platinum sensitivity and overall survival in a cohort of 390 ovarian cancer patients.(27) A Phase II trial of a PARP inhibitor in metastatic prostate cancer also identified somatic and/or germline alterations in a panel of DNA repair genes was significantly associated with response, with 88% of patients who responded to therapy harbored a genetic alteration in an HR DNA repair-related gene.(42) Our results provide direct evidence to support the novel concept that bi-allelic germline and/or somatic alterations in HR genes, rather than the mere presence of a mutation in these genes, lead to phenotypic functional defect in HR and provide a mechanistic basis for these recent clinical observations. Further, we extend the significance of a comprehensive somatic and germline genetic assessment of the HR pathway genes to both the risk and treatment of breast cancer patients.

We were not able to find a clear role for aberrant HR gene expression or BRCA1 promoter methylation in mediating functional HR deficiency in our study. Although, methylation of BRCA1 is enriched in breast cancers compared to normal breast epithelium and leads to

reduced BRCA1 expression, whether these changes have phenotypic consequences remains unclear.(44) In our cohort of breast cancer patients, we only identified two cases with BRCA1 promoter methylation, of which, one case was HR proficient and the other was HR deficient; the latter, however, harbored a homozygous deletion of CHEK2. Hence, we did not find clear evidence that epigenetic alterations in BRCA1 dysregulated HR. In other malignancies, such as ovarian cancer, *BRCA1* promoter methylation occurs in 10–20% of cases and is mutually exclusive of *BRCA1* mutation.(28) Interestingly, though, epigenetic dysregulation of HR in ovarian cancer does not appear to be linked with overall survival or progression free survival after treatment with cisplatin.(45) Ultimately larger cohorts may be required to link epigenetic changes to phenotypic deficiencies in HR.

The only patient with dysfunctional HR who did not have a bi-allelic alteration in a bona fide HR gene, also lacked evidence of a genomic 'scar' or mutational signature consistent with HRD. This is consistent with the notion that dysregulation of the HR pathway may have occurred late in tumor evolution in this particular patient, hence not leaving a mark on the genome. On the opposite end of the spectrum, we identified one tumor with a bi-allelic *BRCA1* mutation without evidence of a functional deficit in HR. This case did not display evidence of intra-genic deletions or reversion mutations in the tumor. Moreover, 53BP1 gene expression was assessed, and levels were not significantly depressed relative to the other samples. Other mechanisms of restoring DNA repair in BRCA1 deficient tumor cells have been reported, such as alterations in *RIF1, HELB, PTIP or MAD2L2*.(46–49) In addition, this case displayed a frameshift mutation in *BRCA1* at the C terminus in the 2nd BRCT domain (Gln1777fs) and also lacked both a high LST score and mutational signature 3. In ovarian cancer, mutations towards the end of the gene have been associated with a worse overall survival (as opposed to mutation in other portions of the gene which are associated with improved survival) – suggesting the possibility that this particular mutation may not necessarily result in an HR deficiency.(50)

Consistent with the notion that genomic 'scars' and mutational signatures are present in breast cancers with HRD, here we demonstrate using a functional HRD test that these genomic 'scars' and mutational signatures are present not only in BRCA1/BRCA2 breast cancers, but also in non-*BRCA1/BRCA2* breast cancers displaying functional HRD. It should be noted, however, that the mutational signature of BRCA1 and/or BRCA2 breast cancers (signature 3 from Alexandrov et al.)(25) seems to identify a more limited subset of HRD breast cancers than the *ex-vivo* RAD51-based functional assessment described here. In addition, genomic 'scar' predictors of HRD only have moderate positive predictive value for functional HRD providing one reason for the modest utility of these assays in clinical trials.(17, 18) Using the finding from our clinical data of a strong relationship between functional HRD and bi-allelic alterations in HR genes, we interrogated the TCGA data to identify cases with bi-allelic alterations in DNA repair genes. As anticipated, we found TCGA cases with bi-allelic alterations had a higher prevalence of genomic scars (i.e. high LST score), providing additional support for our hypothesis that bi-allelic alterations in DNA repair genes mediate HR deficiency in breast cancer.

The results of the functional RAD51 assay described here, in conjunction with other studies(21, 51) highlight the need for a biomarker of HR function to select breast cancer

patients who may benefit from synthetic lethal approaches targeting HRD. Direct testing of induced RAD51 is challenging to implement as a routine clinical test due to the need for fresh tissue, rapid processing, and specialized assessment.(52) In a translational setting, however, functional assessment of the HR pathway can allow for a more thorough interpretation of genomic alterations measured simultaneously. Bi-allelic inactivation of HR genes was found to identify almost 90% of cases with a functional HR defect, with only one false positive result.

This study has important limitations, including the relatively small sample size. Functional *ex-vivo* testing is difficult to perform in a large-scale setting, however, the power of these assays comes from providing a strong readout with which to interrogate genomic data. Furthermore, the findings stemming from our genomic analyses are supported by the reanalysis of a larger cohort of patients from the TCGA., We used research versions of LST and other genomic 'scar' methods rather than the commercial tests, which may slightly alter the performance characteristics described here. Lastly, one of the genes in our *a prior* determined panel of HR genes, *TP53BP1*, is known to regulate pathway choice between HR and NHEJ.(53) In a *BRCA1* mutant background, depletion of *TP53BP1*, rescues an HR defective phenotype. Recent work however, has suggested that in a *BRCA1* wild-type setting, *TP53BP1* is important for adequate RAD51 induction after IR and that exhaustion of *TP53BP1* leads to hyper-resection (and possibly faulty HR).(34) Regardless, the exclusion of this particular case (SP29), does not significantly alter our findings as shown in the sensitivity analysis in the results section.

In conclusion, we identified the genetic basis of HR deficiency in breast cancer by correlating a functional phenotype with bi-allelic genotypic alterations in HR genes. Our results indicate that HR panel gene sequencing would succeed in predicting HR function with almost 90% accuracy. Lastly, our work highlights the importance of having bi-allelic alterations in the HR pathway, as opposed to 'single-hits' to result in a functional deficiency in HR. Comprehensive sequencing of HR genes may allow for a precision medicine approach for DNA damaging therapies and warrants further investigation in large cohorts from prospective clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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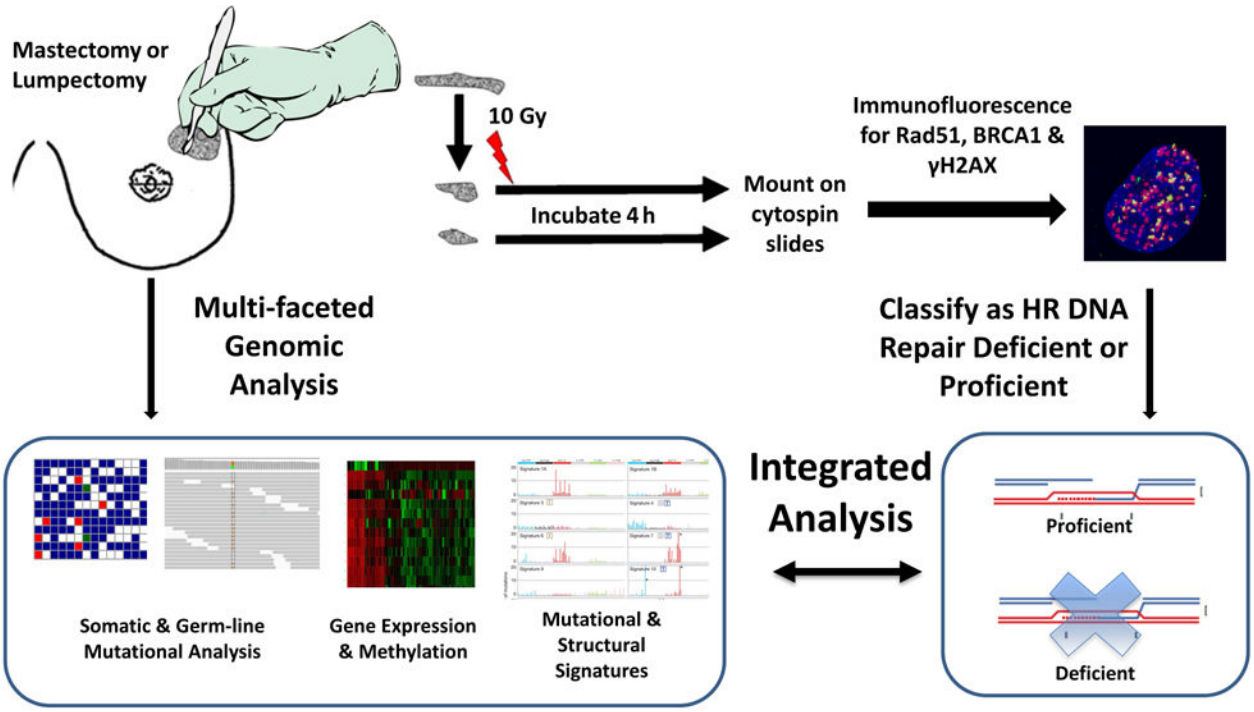

Figure 1. Schematic of study design

Tumors were prospectively collected from 56 patients for *ex-vivo* functional assessment of the status of the HR pathway, using RAD51 foci analysis. Tumors were classified as HR deficient or proficient using this assay. A multi-faceted genomics approach, integrating whole-exome sequencing, analysis of germ-line mutations, copy number variation, gene expression, and methylation was then used to determine the underlying etiology of HRD.

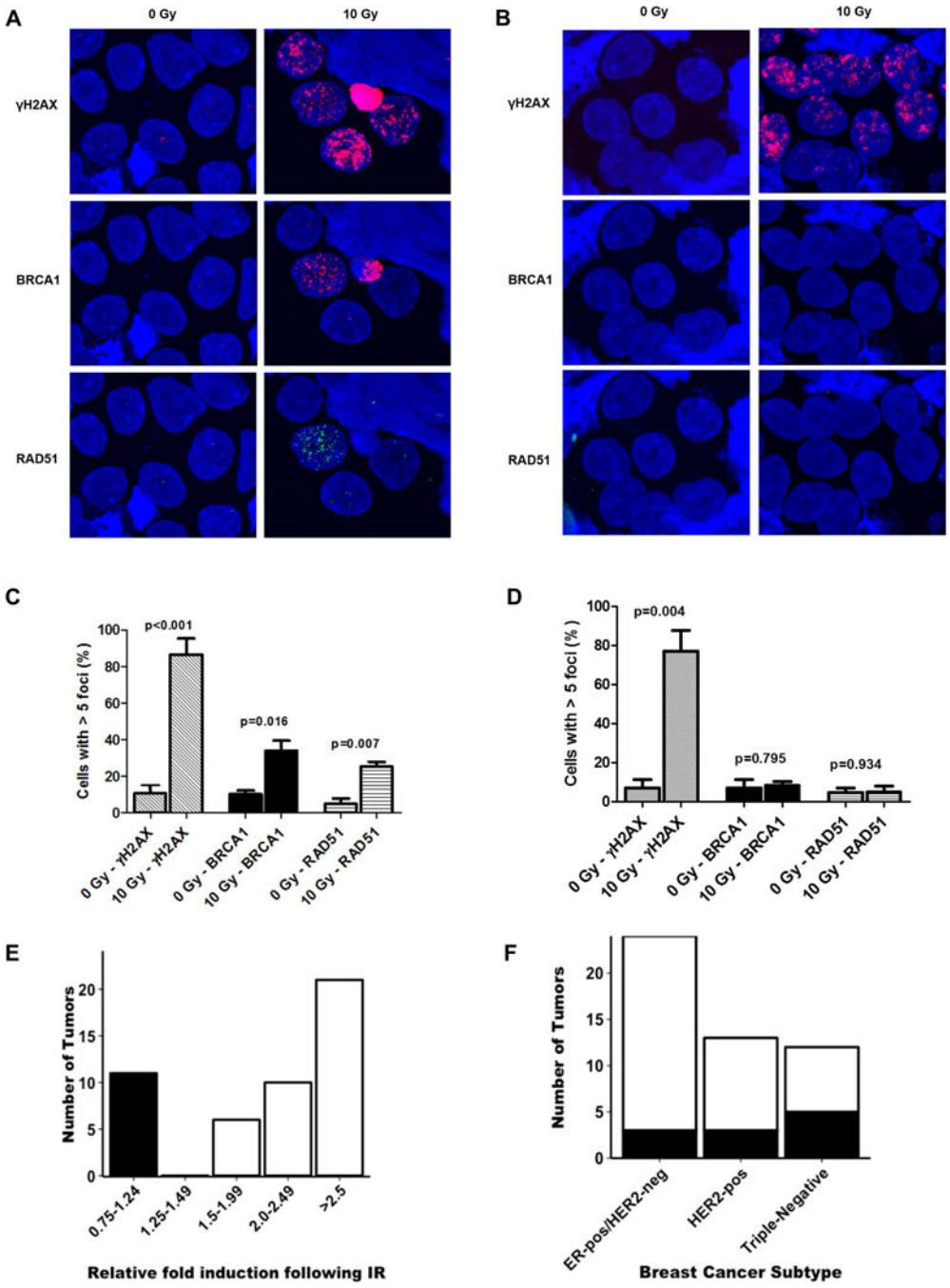

Figure 2. RAD51, γH2AX, and BRCA1 nuclear foci analysis of representative RAD51-proficient and RAD51-deficient case and distribution of RAD51-deficiency in breast cancer

a.) RAD51, γH2AX, and BRCA1 foci in a homologous recombination HR-proficient breast cancer in mock-treated (left) and irradiated conditions (right). b.) Radiation-induced RAD51, γH2AX, and BRCA1 foci in a breast tumor with deficient HR in mock-treated (left) and irradiated conditions (right). c.) Quantification of RAD51, γH2AX, and BRCA1 foci in cells (n=200) from a tumor with proficient HR. Note strong increases in the number of cells with RAD51, γH2AX, and BRCA1 following 10 Gy of ionizing radiation (IR) (error bars indicate s.e.) d.) Quantification of foci in in cells (n=200) from a tumor with

deficient HR. Note strong induction in γH2AX with IR, without an increase in RAD51 or BRCA1 foci. All statistical comparisons were performed by comparing two proportions with a Z-test. e.) Relative fold induction of RAD51 foci formation in the irradiated, compared with the un-irradiated condition for all tumors. The relative fold induction is calculated as the number of nuclei with > 5 foci in the irradiated state divided by the number of nuclei in the un-irradiated state. A bi-modal distribution in relative fold induction is demonstrated, with 11 tumors (black) exhibiting <1.25 fold induction of RAD51 foci and classified as functional HRD. f.) Distribution of RAD51-deficient tumors according to the clinical subtypes of breast cancers. Although RAD51-deficiency was numerically more frequent in triple-negative breast cancers, this was not statistically significant (TNBC, 42%, p=0.13, Fisher's exact test). ER, estrogen receptor; pos, positive; neg, negative.

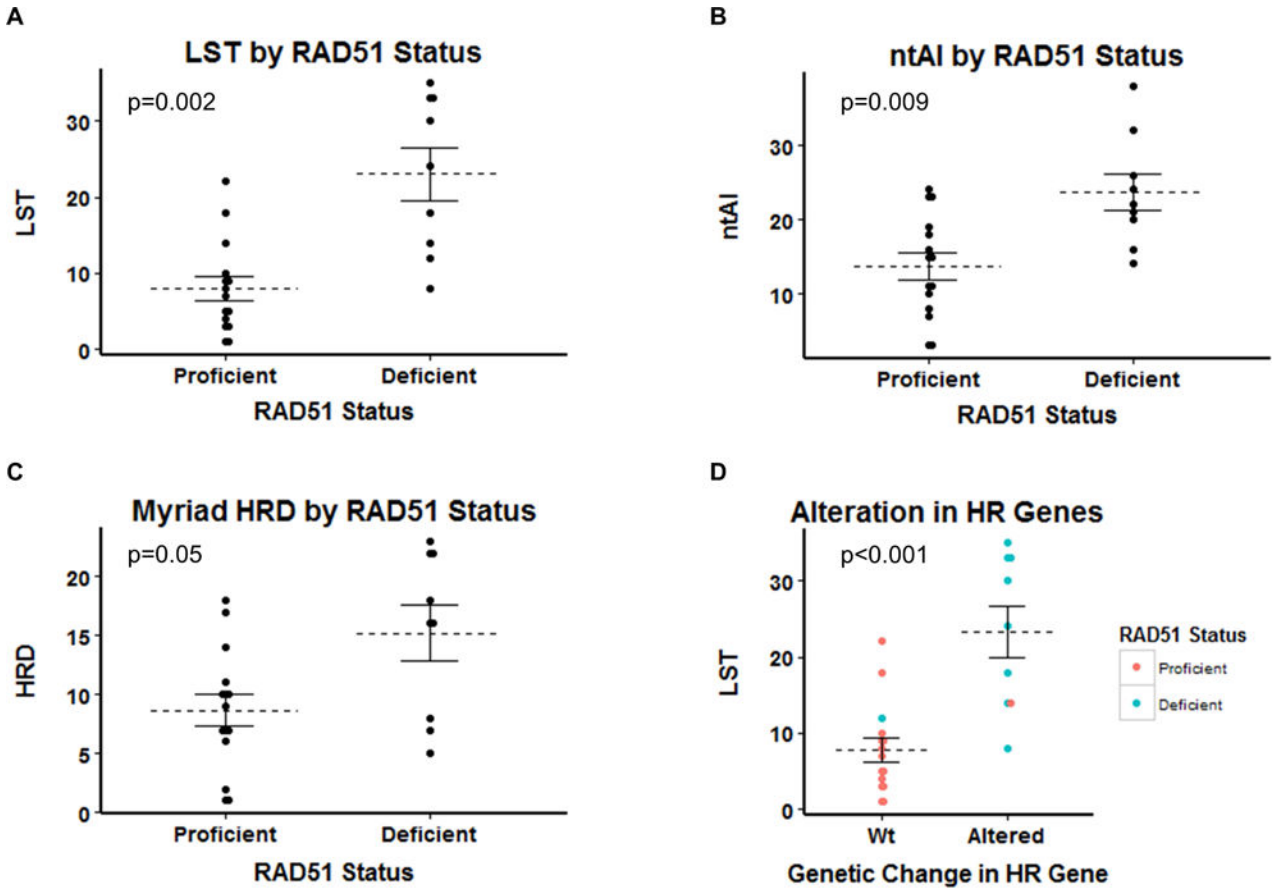

Figure 3. Association of Genomic 'Scars' with RAD51 status

a.) RAD51-deficient breast cancers harbor a higher LST score than RAD51-proficient cases (p=0.002). b.) ntAI scores by RAD51 status in RAD51-proficient and RAD51-deficient breast cancers (p=0.009). c.) RAD51-deficient breast cancers have a higher Myriad LOH/HRD score than RAD51-proficient cancers (p=0.048). d.) Breast tumors with an alteration in an HR Gene (Truncating/frame-shift mutation, homozygous deletion, or non-synonymous mutation with loss-of-heterozygosity) show significantly higher LST scores than those without a genetic alteration in an HR gene ($p = 5.2*10^{-4}$). Wt, wild-type. All comparisons were performed using Wilcoxon rank-sum tests.

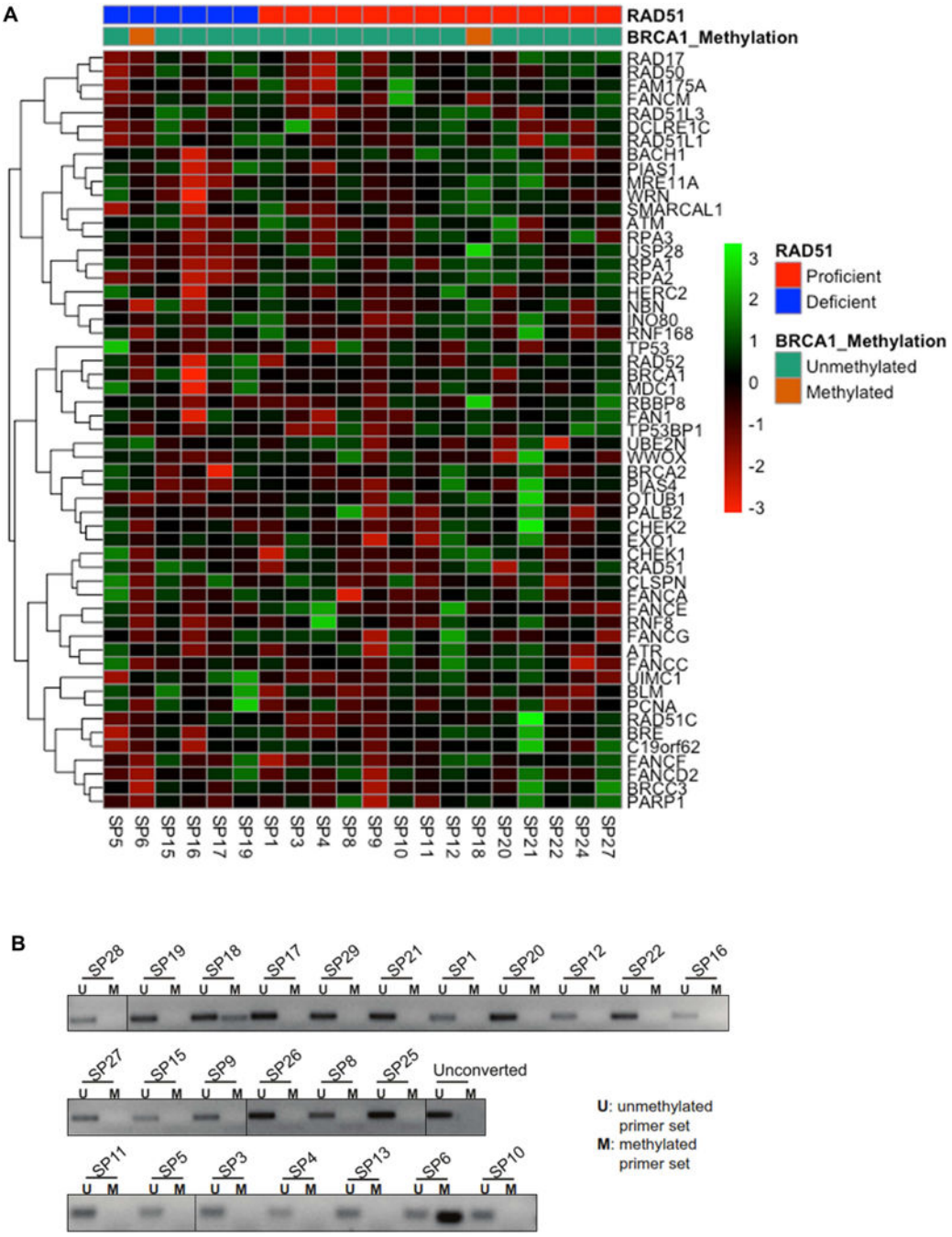

Figure 4. Relationship between RAD51 status and gene expression and methylation

a.) Normalized NanoString expression counts of homologous recombination (HR) DNA repair-related genes compared between DNA repair-deficient (HRD) and DNA repair-proficient tumors as determined by RAD51 foci formation. No individual gene expression was associated with RAD51 status (statistical comparisons performed with t-tests). Supervised hierarchical clustering was unrevealing. Bisulfite sequencing of *BRCA1* promoter using primer sets for un-methylated and methylated PCR is indicated in annotation panel below RAD51 status. Note, data in figure is only shown for samples with both gene

expression and methylation available, however statistical tests were performed with all available data. b.) Bisulfite sequencing of *BRCA1* promoter using primer sets for unmethylated and methylated PCR. The presence of a product in the methylated reaction indicates the presence of methylation in *BRCA1* promoter.

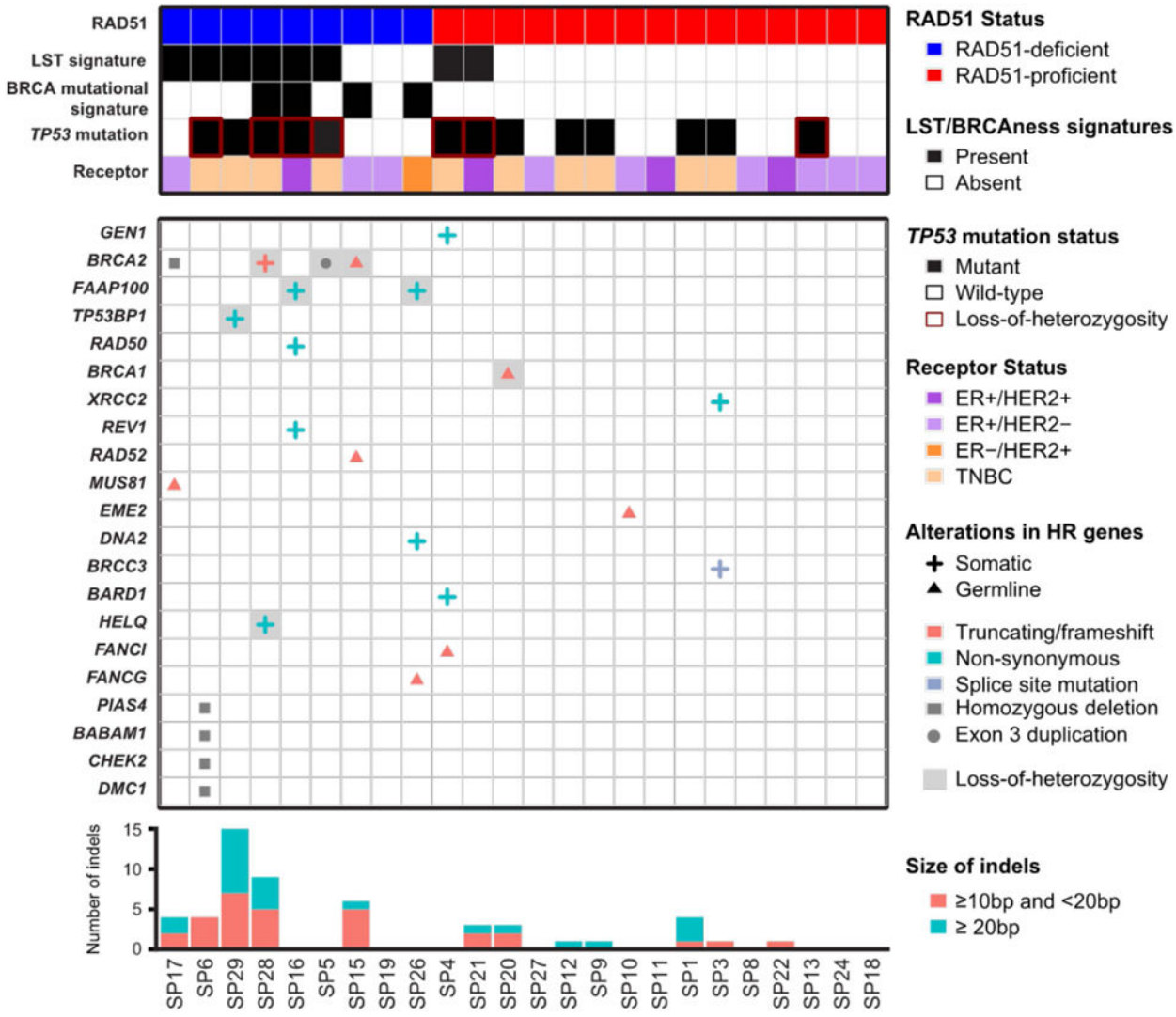

Figure 5. Genetic changes in HR genes in RAD51-deficient and proficient samples

The repertoire of large-scale state transitions (LSTs), the number of somatic insertions and deletions (indels), association with BRCA mutational signature, as well as germline and somatic genetic alterations in genes associated with homologous recombination are presented. Cases are ordered first by RAD51 status, then by increasing LST. The number of indels for each case is divided by size according to the color key. Cases with a BRCA-associated mutation signature are annotated (see Online Methods for details). The grid illustrates the germline and somatic genetic alterations in HR genes. The types of alterations are indicated in the color key on the right. *PIK3CA* and *TP53* mutation status, receptor and RAD51 status, are annotated in the phenobar (top). Exon duplication refers to a duplication of exon 3 in the *BRCA2* gene. ER, estrogen receptor; TNBC, triple-negative breast cancer.

Table 1

Clinico-pathologic characteristics of breast cancer patients included in this study.

Characteristic	No.	%
No. of patients	56	
Age, years		
Median	56	
Range	20–81	
Sex		
Female	55	98
Male	1	2
Menopausal Status *		
Pre-	25	45
Peri-	1	5
Post-	28	50
Family history		
First Degree	12	21
Second Degree	15	27
Surgery		
Mastectomy	25	45
Lumpectomy	31	55
ER and/or PR positive	30	54
HER2 amplified	14	25
Triple negative	12	21
Tumor Size		
Median	2.5	
Range	1.1–6.5	
Histologic Subtype		
Invasive ductal carcinoma	51	91
Invasive lobular carcinoma	5	9
Histologic Grade **		
1	0	0
2	7	14
3	44	86
Nuclear Grade **		
1	1	2
2	18	35
3	32	63
Node positive	33	59
≥4 positive nodes	15	27

Characteristic	No.	%
Proliferation Index		
Adequate	49	87.5
Inadequate	7	12.5

*1 male breast cancer patient not included

**Includes only invasive ductal carcinomas

Table 2

Association of clinical features with homologous repair deficiency as defined by RAD51 status. Comparisons of age and median tumor size were performed using unpaired t-test. Other statistical comparisons were performed using Fisher's exact-test.

Patient Clinical and Pathologic Characteristics Stratified by Homologous Recombination Function					
Characteristic	**Repair Deficient**	**%**	**Repair Proficient**	**%**	***P* value**
Patient number	11		38		
Median Age	49		56		0.23
Family History *	9	82	17	45	0.04
Premenopausal **	5	45	18	47	1
Median Tumor Size, cm	3		2.5		0.24
Node Positive	5	45	24	63	0.49
≥4 positive nodes	3	27	8	21	0.69
Histologic Subtype					
Invasive Lobular Carcinoma	0	0	4	11	0.56
Invasive Ductal Carcinoma	11	100	34	89	
Histologic Grade 3 ***	10	91	30	79	0.66
Nuclear Grade 3 ***	10	91	22	58	0.07

* First or Second degree

** 1 male breast cancer patient excluded

*** Includes only invasive ductal carcinomas