A mutational signature reveals alterations underlying deficient homologous recombination repair in breast cancer

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Abstract

Biallelic inactivation of *BRCA1/2* is associated with a pattern of genome-wide mutations known as Signature 3. By analyzing ~1,000 breast cancer samples, we confirmed this association and established that germline nonsense and frameshift variants in *PALB2*, but not *ATM* or *CHEK2*, can also give rise to the same signature. Missense *BRCA1/2* variants known to impair homologous recombination (HR) were accurately classified by this signature. Finally, we show that epigenetic silencing of *RAD51C* and *BRCA1* by promoter methylation are strongly associated with Signature 3 and, in our dataset, were highly enriched in basal-like breast cancers in young patients of African descent.

INTRODUCTION

Breast cancer is the most common non-cutaneous malignancy and approximately 10–15% of cases are associated with hereditary syndromes of defective DNA repair[1]. The most common forms of hereditary breast and ovarian cancer are associated with pathogenic germline variants in two homologous recombination (HR) repair genes, *BRCA1* and *BRCA2*[2]. HR is an evolutionarily-conserved pathway that coordinates high-fidelity repair of double-stranded DNA breaks (DSBs), and functions primarily during the late S and G2 phases of the cell cycle to exploit the intact sister chromatid as a template for error-free repair[3]. Although germline alterations that abrogate the tumor suppressor functions of *BRCA1* and *BRCA2* play a role in breast cancer pathogenesis, comprehensive sequencing studies have demonstrated that somatic mutations and epigenetic events in these genes can also drive tumorigenesis[4–7]. Deficiency of HR leads to cellular dependence on alternate error-prone DNA repair pathways, yielding characteristic genomic alterations, higher overall mutational rates, and unique dependencies used for therapeutic targeting[8].

Analyses of tumor-derived genome sequences have revealed that loss of *BRCA1* or *BRCA2* yields a distinct pattern of base-substitution mutations, termed "Signature 3" [9,10]. This pattern, among others, can be discerned using non-negative matrix factorization (NMF), a technique used to identify recurring patterns (i.e. signatures) in the spectra of mutations from a set of tumors and to estimate the contributions of these signatures to the mutational landscape of the individual tumors. The etiology of some signatures was revealed by associating their activities with additional data[10–13]. While recent efforts have observed a significant association between *BRCA1/2* loss and Signature 3[9,10], several tumors exhibit high levels of Signature 3 without discernible alterations in HR-pathway genes[9,10]. Furthermore, Signature 3 has not been extensively correlated with other genetic and epigenetic events in HR genes. A comprehensive understanding of the link between HR

pathway and Signature 3 is of clinical relevance given the implications for underlying cancer risk and treatment selection.

Current clinical practice in testing for breast cancer susceptibility relies largely on identifying known pathogenic variants in the germline sequence of *BRCA1/2* and a limited number of related genes[14,15]. Such germline testing may uncover germline pathogenic variants that are likely to contribute to HRD in the associated breast tumor. The presence of HRD may expose specific therapeutic vulnerabilities. We, therefore, wanted to assess whether integrating germline and somatic data can improve the detection of HRD. Somatic events, such as loss-of-heterozygousity, epigenetic silencing and other mechanisms that can decrease gene expression, can serve as the "second-hit" in these genes.[16]. Moreover, integrating somatic data can help interpret poorly-defined germline variants[17]. Here, we hypothesize that Signature 3 could represent a more reliable readout of HR status than germline sequencing alone and could enhance prediction of breast cancer risk. Moreover, Signature 3 may aid in classifying variants that have yet to be functionally characterized. We, therefore, sought to identify the mechanisms by which Signature 3 could arise in the absence of pathogenic germline alterations in *BRCA1/2*. Specifically, we analyzed the association between Signature 3 and multi-dimensional events (germline variants, somatic mutations, epigenetic silencing, and allelic imbalance) in HR pathway components aside from *BRCA1/2*.

A distinct mutational signature is associated with biallelic inactivation of BRCA1/2 and epigenetic silencing of BRCA1

We first characterized the underlying landscape of genomic lesions operating in breast cancer by performing mutational signature analysis of 995 invasive breast carcinoma samples from The Cancer Genome Atlas (TCGA)[4]. We stratified single-nucleotide variants (SNVs) by base substitutions in 96 possible tri-nucleotide contexts (Figure 1, top row "SNVs") and applied our method, SignatureAnalyzer[12,13], which users a Bayesian derivative of NMF to infer the number of operating signatures as well as the activity of each signature in each tumor (Methods).

We identified seven distinct mutational signatures across the 995 TCGA breast cancers, largely corresponding to previous analyses of this series[10] (Supplementary Figure 1 and Methods). Of these, three signatures arose each in a single case, which we excluded from subsequent analyses (Methods), thereby yielding four recurrent signatures across 992 samples (Figure 1; Supplementary Figure 2): (i) Signature 1 (C>T in CpG sites); (ii) an APOBEC related signature (combination of Signature 2 and Signature 13); (iii) a signature associated with microsatellite instability (Signature 6); and (iv) a largely uniform signature similar to the previously reported Signature 3, which was associated with mutations in *BRCA1/2*[9,10,18].

Over 10% (100/992) of the samples harbored clinically-relevant germline or somatic frameshift/nonsense variants in *BRCA1/2*, well-characterized pathogenic missense *BRCA1/2* germline variants[19] and/or epigenetic silencing of the *BRCA1* promoter[16]. As previously demonstrated, a variety of *BRCA1/2* inactivating variants are associated with Signature 3 (Figure 2; Supplementary Figure 3)[9,10,18]. Fifty samples with somatic or

germline nonsense/frameshift variants in *BRCA1*/2 exhibited a ~6-fold increase in levels of Signature 3 in comparison to samples without *BRCA1/2* alterations (p <0.001, rank sum test, Figure 3A), consistent with the enrichment of Signature 3 reported in a recent data set[9] (Figure 3B). Aside from single nucleotide variants, epigenetic silencing of *BRCA1* (n = 32) and homozygous deletions of *BRCA1/2* (n = 8) were also associated with Signature 3 (Figure 3A). Moreover, 18 samples harboring the lower-risk truncating *BRCA2* variant p.Lys3326Ter[20] (c.9976A>T, chr13:g.32972626A>T (hg19);NM_000059.3) did not exhibit elevated levels of Signature 3 (median of 13.4 Signature 3 mutations/sample; p = 0.4; data not shown).

A single functional copy of *BRCA1/2* is generally sufficient to maintain normal HR function while biallelic inactivation of *BRCA1/2* contributes to tumorigenesis by several mechanisms, including genomic instability that arises from HR deficiency (HRD)[21]. To investigate whether monoallelic loss of *BRCA1/2* is associated with Signature 3, or whether HRD (biallelic loss) is necessary for Signature 3, we analyzed loss of heterozygosity (LOH) at the *BRCA1/2* loci (Methods), as these are the most common "second-hit" events[22,23]. We identified 23 samples with LOH in which the retained *BRCA1/2* allele contained a germline frameshift/nonsense variant while the intact allele was lost (Supplementary Figure 4). All samples but one exhibited Signature 3 activity in the top quartile for the series (Figures 2, Supplementary Figure 4; $p = 8 \times 10^{-15}$; median of 68.9 vs. 9.7 for no *BRCA1/2* events). Similarly, all 18 samples with LOH that also harbored a somatic frameshift/nonsense/splice-site *BRCA1/2* variant had high Signature 3 activity (Supplementary Figure 4; $p = 2.8 \times 10^{-10}$).

In contrast, monoallelic germline inactivation of *BRCA1/2* was not associated with Signature 3 (Supplementary Figure 4), consistent with recent reports[9]. Of 5 samples harboring pathogenic germline variants without loss of the intact allele, 4 showed no increase in Signature 3 activity (p = 0.42). Two samples with a somatic truncating mutation and without evidence of biallelic inactivation also failed to show Signature 3 elevation (Supplementary Figure 4). RNA-seq data for these 5 cases had very low coverage of *BRCA1/2* at the variant site (2 reads), and could not be used to examine expression of the mutant *vs.* wildtype alleles. In addition, samples with LOH of *BRCA1/2* that retained an allele with a benign missense germline variant did not exhibit strong enrichment of Signature 3 (Supplementary Figure 4; median of 12.9 vs 68.9 where LOH retained a truncating event). These findings suggest that Signature 3 may be a reliable readout of biallelic, and not monoallelic, loss of *BRCA1/2* and/or of HRD.

Despite the association between *BRCA1/2* inactivation (germline, somatic or epigenetic silencing) and Signature 3 (88 of 100 *BRCA1/2* events were in the top quartile of Signature 3 activity), most samples in the top quartile (159 of 247) did not have such events, suggesting that other lesions might contribute to Signature 3 activity. Clinical assays for the assessment of breast cancer risk include gene panels of varying breadth, nearly all of which include a variety of DNA repair genes. As discussed above, numerous germline variants of *BRCA1/2* confer high cancer risk, however, deleterious variants in several other genes confer "moderate risk"[15] (Supplementary Figure 5). Therefore, we sought to determine whether alterations in these latter genes are associated with Signature 3 (Figure 3C), with a

particular focus on germline events in which the intact allele was lost (Supplementary Table 1; Supplementary Figure 6). Below, we consider the association between deleterious variants in these genes and Signature 3.

Signature 3 is associated with lesions in established breast cancer gene PALB2

In search of additional germline variants that could be associated with Signature 3, we examined all 992 cases for any deleterious alterations in other known genes of the HR pathway, including *PALB2*, a binding partner and nuclear localizer of BRCA2 (Supplementary Figure 6). In recent studies, germline truncating variants in *PALB2* have been associated with a relative risk for breast cancer between 3.4 and 6.6[15,24,25]. Three samples among our cohort harbored germline nonsense/frameshift variants in *PALB2,* and all exhibited elevated Signature 3 activity (Figure 3C). We could not validate this association in a recent report of 560 breast cancers[9] due to lack of these germline events. Consistent with our results, however, in a recent pancreatic study[26,27], two cases with *PALB2* germline truncating variants had high levels of Signature 3.

RAD51C methylation as a key alteration underlying deficient homologous recombination repair in basal-like breast carcinoma

RAD51 is involved in DNA repair by HR, promoting DNA strand invasion and homology search[14]. This process requires RAD51 in complex with BRCA2 and, to a lesser extent, PALB2[28], both of which can promote RAD51 loading onto single-stranded DNA. There are several *RAD51*-related genes, including *RAD51B*, *RAD51C*, *RAD51D*, *DMC1*, *XRCC2* and *XRCC3*. RAD51C facilitates the accumulation of RAD51 to sites of DNA damage by complexing with several RAD51-like proteins (Figure 4A).

It is generally thought that germline truncating variants in *RAD51C* do not confer an increased risk of breast cancer[29], although widespread panel-based genetic testing has revealed deleterious *RAD51C* variants among 0.3% of triple negative breast cancer patients[30] and in high-risk breast pedigrees[31]. In our data, one case displayed biallelic inactivation of *RAD51C* (a germline variant with LOH that deleted the intact allele), and another case presented somatic homozygous deletion. Both tumors exhibited high levels of Signature 3 (Figures 2 and 3C), suggesting that inactivation of *RAD51C* can lead to HRD.

As *BRCA1* promoter hypermethylation can promote breast cancer development, we interrogated whether such events also occur in other HR genes. Indeed, *RAD51C* promoter methylation correlated with downregulated gene expression in our series (Figure 4B). Of 23 cases of *RAD51C* promoter methylation, 19 showed 2-fold decrease in mRNA expression (Figure 4B). Seventeen of the 23 samples were in the top quartile of Signature 3 activity and all but one did not have any *BRCA1/2* event (the one with a *BRCA1/2* event had elevated *RAD51C* expression). Thus, 16 cases of *RAD51C* epigenetic silencing explain 10% (16/159) of the top quartile without *BRCA1/2* events (Figure 4C). Moreover, the number of samples with high Signature 3 that harbor epigenetic events in *BRCA1* and *RAD51C* is comparable to the number of germline events in *BRCA1/2,* suggesting a similar contribution of germline and epigenetic events to carcinogenesis (Figure 4C).

Of note, 52% (12/23) of samples with *RAD51C* silencing exhibited the basal-like expression pattern and all of these basal-like cases were among the 17 *RAD51C*-silenced samples in the top Signature 3 quartile (70%; p = 0.003, Fisher's Exact Test). Examining an independent series[32,33] with methylation and expression data showed a similar correlation between promoter methylation and expression of *RAD51C* (Supplementary Figure 7). Consistent with our findings, cases with *RAD51C* promoter methylation and low expression levels were highly enriched with basal-like tumors (Figure 4B; Supplementary Figure 7). Basal-like expression is characteristic of breast cancers with *BRCA1* defects[34], seen in 81% (17/21) of cases with germline *BRCA1* deleterious variants and in 81% (25/32) of cases with *BRCA1* promoter hypermethylation[35,36]; the last association was also observed in the independent series (Supplementary Figure 7). In contrast, only 10% (2/20) of tumors with *BRCA2* germline deleterious variants were basal-like, suggesting that inactivation of *RAD51C* may be biologically closer to cases with *BRCA1* defects rather than to those with *BRCA2* inactivation. *RAD51C* promoter methylation was only associated with increased level of Signature 3 in basal-like tumors (Figure 4D), consistent with the strong reduced expression in this subtype, suggesting that *RAD51C* promoter methylation plays a role in HRD mainly in the context of basal-like tumors.

No association of Signature 3 with mutations in DNA damage signaling pathway genes

The role of *BRCA1/2* in PARP inhibitor susceptibility is established. However, the therapeutic significance of deleterious variants in HR components upstream of BRCA1/2 (Supplementary Figure 5), such as ATM, NBN and CHEK2, that are also implicated in DNA damage signaling and double-strand break detection and increase the risk of breast cancer[15] (relative risk [RR] 2–3), is less clear. In our dataset, we identified 17 germline pathogenic variants in six established or candidate breast cancer susceptibility genes (7 in *ATM*, 4 in *CHEK2*, 3 in *NBN* and one in *BARD1*, *BRIP1* and *RAD50*; see Supplementary Note for discussion). Overall, germline pathogenic variants in *ATM* and *CHEK2* were not associated with a high level of Signature 3 (Supplementary Table 1; Figure 3C) and this result was replicated in the previously published dataset[9] (Figure 3B). The single case with *BARD1* truncating variant and LOH (losing the wild-type allele), had a high level of Signature 3 (Figure 3C and Supplementary Figure 6).

Promoter methylation events of BRCA1 and RAD51C are enriched among young black patients

We next sought to assess whether self-reported racial differences underlie various Signature 3 etiologies. The largest racial groups within our series consisted of 699 white patients and 142 black/African American patients (the remaining 181 included 57 Asian patients). We evaluated differences in the frequencies of genetic (germline and somatic) and epigenetic events that contribute to Signature 3 between these racial groups, focusing on the cases in the top quartile of Signature 3 activity (159 white and 41 black/African American).

Among the 41 black patients in the top quartile, 36% (15/41) showed promoter methylation in either *BRCA1* or *RAD51C*, 9.8% (4/41) harbored germline or somatic deleterious mutations in *BRCA1/2* and the remaining 22 had no discernible lesion (Figure 5A). In contrast, among the 159 white patients in the top quartile, 13%, (22/159) exhibited an

epigenetic event, while 32% (56/159) had germline events or somatic deleterious events and the 81 remaining cases had no event (Figure 5A, Freeman-Halton 3×2 exact test p = 3×10^{-4} for whites compared to blacks). Reduced mRNA levels of *RAD51C* and *BRCA1* in cases with promoter methylation were similar among white and black patients, suggesting similar function effects.

Among premenopausal women, African Americans are at higher risk of developing basal-like breast cancers than their Non-Hispanic White counterparts[37]. Given the association between HR-deficiency and basal-like biology, and the observed differences between racial groups in the prevalence of Signature 3-associated genetic and epigenetic events, we further investigated differences by age subgroups (Figure 5B) and within the basal-like subtype (Figure 5C). The most pronounced differences arose among women aged 40–49 years. Of black patients in this age group, 80% (8/10) exhibited promoter methylation (6 *BRCA1* and 2 *RAD51C*) in contrast to only 18% (7/38) of white patients. Conversely, genetic events were enriched among white women (36%; 14/ 38), while only one black woman in this age group had a genetic alteration (1/10). This suggests that the mechanism of HR deficiency differs between white patients, in whom genetic mutations predominate, and black patients in whom promoter methylation is more frequent (p = 0.0009). We further examined if this difference also arises in basal-like patients aged 40–49 and found a similar disparity (Figure 5C, p = 0.0065).

Signature 3 activity correlates with the pathogenic potential of rare germline variants

We next sought to evaluate whether Signature 3 could be used to characterize rare missense SNVs in *BRCA1/2*. Understanding the pathogenicity of rare germline variants of *BRCA1/2* has become the focus of multiple international consortia[38,39] and has led to the assignment of consensus annotations to rare variants into five classes: benign, likely benign, variants of uncertain significance (VUS), likely pathogenic and pathogenic[40]. Over the entire series of 992 cases, 262 (26.3%) harbored at least one rare (prevalence <0.1%) missense SNV in *BRCA1/2*.

As discussed above, the biallelic inactivation of *BRCA1/2*, particularly via an inherited pathogenic variant and LOH is considered a hallmark of HRD. As shown above, nearly all samples with pathogenic *BRCA1/2* germline variants and loss of the intact allele exhibited high levels of Signature 3, while those harboring pathogenic variants without LOH had no elevation of Signature 3. Conversely, samples with LOH that otherwise retained an allele harboring a silent germline variant were not associated with a significant increase in Signature 3 (Supplementary Figure 4), thereby suggesting that the presence of LOH alone cannot accurately identify samples with HRD. Signature 3 in conjunction with LOH, however, could represent a robust readout of HRD and of the pathogenic potential of indeterminate variants. As a result, we sought to apply a "bi-allelic inactivation" model with high Signature 3 levels to potentially discriminate between pathogenic and benign variants. We estimated the likely pathogenicity of a variant by employing two methodologies based on the bi-allelic inactivation model for HRD (Methods).

Of the 262 cases discussed above, we observed 143 distinct missense germline SNVs in *BRCA1/2*, 74 were present in >1 sample, of which 8 also exhibited LOH in at least two

tumors by losing the intact allele (Supplementary Table 2). Of these 8, one is considered pathogenic and 7 are thought to be benign[19]. The pathogenic variant, BRCA1, p.Cys61Gly (Supplementary Table 2 for HGSV nomenclature), was present in four samples, all of which exhibited high Signature 3 activity, and biallelic inactivation (3 with LOH, 1 with an additional somatic truncating variant, p-value = 0.004, Supplementary Table 2). p.Cys61Gly in BRCA1 is a founder variant among Poles and disrupts the RING-domain interaction between BRCA1 and BARD1[41,42]. Here, it was identified in a largely unbiased approach by seeking rare germline missense SNVs with concomitant LOH, without familial, pathological or other clinically-relevant data. The remaining 7 germline variants, previously described as benign, were not associated with an increase in Signature 3 activity.

We hypothesized that LOH status in tandem with Signature 3 might further inform the clinical implications of poorly-characterized *BRCA1/2* germline variants. To test this approach, we used the set of germline missense SNVs in our data for which a functional implication had been classified by the ENIGMA consortium (n=142): 5 as pathogenic variants (class 5 in ClinVar) and the remainder 137 as benign (class 1)[39] (Figure 6). Therefore, in our dataset the fraction of pathogenic missense variants was 3.5% ($CI_{95\%}$ [1%, 8%]). However, among samples with germline variants in which allelic imbalance favored the alternate allele, 22% harbored pathogenic variants (4/18; $CI_{95\%}$ [6%, 47%]; p <0.001 by Hypergeometric test). Overlaying the yet more stringent requirement that a variant exhibit both allelic imbalance and high Signature 3 activity (top quartile) yielded a set in which 66% of samples harbored pathogenic alleles (4/6, $CI_{95\%}$ [22%, 99%]; $p < 4.1 \times 10^{-6}$; Figure 6); one of the two apparently-benign variants also harbored *RAD51C* hypermethylation, which can underlie the high Signature 3 activity. By considering both LOH and Signature 3 activity, the likelihood of pathogenicity for a given allele may be improved considerably. Conversely, among 16 samples with an excess of the alternate allele, but with low levels of Signature 3, all *BRCA1/2* variants were benign, suggesting that this method is not only sensitive, but also highly specific. Moreover, in an independent dataset of whole genomes[9], we observed that two pathogenic variants (class 5) and one likely pathogenic (class 4) variant (Figure 3B) had high levels of Signature 3 concomitant with an LOH event that favored retention of the mutated allele [9], thereby demonstrating the sensitivity of this approach, independent of method or platform.

Considering that either LOH or a second deleterious mutation that results in biallelic inactivation, our approach captured the known pathogenic *BRCA1/2* mutations among the series and accurately classified *BRCA1/2* VUSs. Overall, 12 unclassified missense variants fulfilled the criteria (Supplementary Note). Downstream, only 3 potentially pathogenic rare missense mutations had supportive evidence (Supplementary Table 3). Thus, in a dataset of ~1000 breast cancers, only 8 rare missense SNVs (of which 5 were previously known) are likely to have contributed to the development of HR-deficient tumors. A similar approach was applied to somatic events and suggested that 4 somatic missense mutations (all in *BRCA1*) may disrupt critical functions within the HR pathway (Supplementary Note).

Signature 3 is a robust and independent biomarker of HR deficiency

Finally, since Signature 3 could be associated with the degree of HRD, we sought to establish a clinically-applicable threshold for binary phenotypic classification. Such a task is difficult since there is no standard to unequivocally establish if HRD is present. However, germline events in *BRCA1/2* and *PALB2* are known contributors to HRD. To standardize a measure to estimate the predictive power of Signature 3 for identifying samples with events in HR pathway genes, we calculated the Area Under the ROC Curve (AUC) using all cases with deleterious alterations in *BRCA1/2*, *PALB2* and *RAD51C* as a ground truth positive set. The AUC for detecting deleterious alterations was 0.86 and increased to 0.93 when considering, as the ground truth set, only germline cases with a second hit (Figure 7A). A similar AUC for classifying HRD events was recapitulated upon using whole-genomes or only 8 MB of sequence, and applying a normalized version of Signature 3. In addition, Signature 3 levels were not correlated with purity in our TCGA series (Supplementary Note, Supplementary Figures 8–11). Hence, Signature 3 activity may serve as a robust classifier of HR events across different platforms.

Assays for HRD are used to identify samples with underlying events beyond germline lesions in *BRCA1/2,* e.g. somatic events in HR genes or epigenetic silencing. The calculated AUC using all events is 0.89 (Figure 7A); slightly higher than that calculated based on *BRCA1/2* germline events, but lower compared to the estimated germline+LOH AUC. These results improve upon alternate approaches that are based on LOH[43] and large scale transition[44] scores (referred to as HRD-LOH and HRD-LST, respectively) (Figure 7B), largely because Signature 3 is more sensitive in identifying *BRCA2* and *RAD51C* events (Supplementary Figure 12). We found that a threshold of 31 Signature-3-associated mutations (chosen to maximize Youden's Index[45]) detects 82% of the events in our dataset that can give rise to HRD and may identify another 11% of samples with a yet unknown defect in HR (Supplementary Figure 13). Of samples above this 31-mutation threshold, we found a putatively-etiologic lesion in roughly one-half. Most, however, also harbored high HRD-LOH and LST scores (Figure 7C), suggesting elevated degrees of genomic instability. To classify bi-allelic inactivation of *BRCA1/2* events the optimal threshold is 37 Signature-3-associated mutations (Supplementary Figure 14; Supplementary Note). Since clinical applications often assay a subset of genes, we tested how many genes would yield an accurate classification of high Signature 3 patients (as defined by exome sequencing) and found that with 1600 genes we can achieve an AUC of ~0.82 (Supplementary Figure 15; Supplementary Note).

DISCUSSION

Our analysis represents an extended characterization of Signature 3[9,10,18] and its relationship to the functional underpinnings of the HR repair machinery. Using a multi-dimensional approach, we demonstrate that this signature is reliably detected among samples with deleterious germline and somatic mutations in *BRCA1* and *BRCA2*. Consistent with recent results[9], we demonstrate and quantify the association of biallelic inactivation of *BRCA1/2* with Signature 3 and show lack of association in cases with mono-allelic inactivation.

We further used Signature 3 to identify a large subset of tumors (16%) with no discernible canonical HR defect, yet with a mutational landscape similar to that of samples with HRD. Focusing on this subset, we found that a small number of them harbor rare truncating variants in other components of the HR machinery.

Most importantly, we demonstrated that epigenetic silencing and somatic mutations in *RAD51C* have similar potential to abrogate HR function and yield the same characteristic mutational signature. The *RAD51C* promoter methylation events are comparable in frequency to *BRCA2* germline events and higher than somatic truncating *BRCA1* events in these tumors. *RAD51C* silencing events are even more frequent in basal-like breast cancers. To emphasize the magnitude of this finding, while *PALB2* is now considered to be an important determinant for hereditary breast cancer[15], we found only 2 samples harboring germline *PALB2* lesions among the top quartile of Signature 3 activity, in contrast to 18 with somatically-acquired *RAD51C* dysfunction, thus making *RAD51C* methylation an important determinant of basal-like breast tumors. Preclinical models lacking *RAD51C* have been reported to exhibit sensitivity to PARP inhibitors[46,47], raising potential translational implications for this finding. Therefore, *RAD51C* methylation could aid in patient stratification for agents that target HRD, as is currently being employed among ovarian cancer patients[48].

In contrast to *BRCA1/2*, *RAD51C* and *PALB2*, protein-truncating germline variants in *ATM*, *CHEK2* and *NBN*, that were shown to confer increased risk of developing breast cancer[15] (including established pathogenic variants, Supplementary Table 1), were not associated with Signature 3. This finding suggests that these events may drive cancer risk via mechanisms independent of the BRCA1/2 double-strand break repair pathway. Indeed, in two pancreatic cancer studies with 17 combined cases of *ATM* mutations, 16 of which were biallelic, only one showed a signature consistent with HRD[26,27]. In contrast, alterations among HR components that are in the same protein complex with BRCA1/2 (e. g. RAD51C, PALB2 and BARD1) are associated with Signature 3, accounting for 8% of samples in the top quartile of Signature 3 activity and 13% of cases without *BRCA1/2* events (Figure 4C). These findings may further extend the rational use of novel therapeutics.

"BRCAness" refers to HRD in a tumor that lacks a germline *BRCA1* or *BRCA2* deleterious variant[16]. As we have shown, Signature 3 can serve as a novel readout of HRD, and as such contribute to BRCAness, likely reflecting other defects in the *BRCA1/2* part of the pathway. It will be important to test the association between different measures of BRCAness including ones based on expression, mutational signatures and other molecular measures, and responses to treatment in clinical trials. Our measure of Signature 3 could represent a putative biomarker for HR status. Current predictors of PARP-inhibitor or cisplatin sensitivity have had notable clinical success in a limited number of patients[49–51], indicating the need for additional biomarkers such as HRD[49] or Signature 3 to identify other patients who could also benefit from these treatments.

Developing a companion diagnostic based on this work could inform appropriate allocation of HRD-based therapies. Genomic-based scores, especially when based on clonal mutations, reflect the aggregated effect of HRD throughout the development of the cancer, but not

necessarily its current HRD status[52] Although not stated, ovarian cancers with *BRCA1* reversion[52] likely retained high levels of Signature 3 inherited from their ancestor cells, and were unlikely to have ongoing HRD. We expect a similar phenomenon with promoter methylation, whereby platinum (or PARP-inhibition) resistance might arise due to demethylation of the relevant promoter (e.g. *BRCA1* or *RAD51C*).

Our findings have significant implications for the analysis of germline variants. When a pathogenic *BRCA1/2* germline variant is found in the setting of a breast or ovarian cancer, it is inferred to be the putative driver of carcinogenesis[53]. However, among 28 carriers of pathogenic germline *BRCA1/2* variants in our series whose tumor LOH status was discernible, we identified 4 tumors that exhibited neither loss of the intact *BRCA1/2* allele nor elevated Signature 3 activity. In these, the tumors either arose independently of BRCA1/2 inactivation, or *BRCA1/2* haploinsufficiency triggered events that, while cancer-initiating, were insufficient to independently generate HRD. BRCA1 haploinsufficiency in breast epithelium can lead to defective repair of stalled replication forks[54]. Inactivation of *BRCA2* among other HR factors may cause similar stalling. As these forks can be repaired by non-HR dependent mechanisms[55], *BRCA1/2* haploinsufficiency could lead to non-HRD tumor phenotypes. Consequently, integrating Signature 3 with *BRCA1/2* germline testing, may affect treatment selection.

Moreover, the classification of missense germline variants remains a challenging prospect given the breadth of uncommon variants and the large sample size often needed to reliably determine pathogenicity. Tumor signatures could be a powerful adjunct to existing methods in determining therapeutic approaches[56]. Importantly, our findings must be interpreted in the context of the TCGA study design. Namely, the use of samples from the TCGA may have limited generalizability to underrepresented minorities from whom few samples were collected. Similarly, accessibility to TCGA-participating centers and unblinded enrollment in this study may have introduced unforeseen biases into the study cohort. The clinical utility of Signature 3 will require prospective validation given the scarcity of pathogenic missense variants in this cohort (n = 5).

While clinical genetics has long relied on the use of population-level data and family-based segregation analysis to identify high-risk patients, this approach is of somewhat limited utility among those who are part of small pedigrees or who otherwise have limited access to familial information, and in the context of the discovery of increasing numbers of very rare, possibly unique variants. In the near future, with the increasing amount of tumor sequencing data becoming available[57], mutation signatures extracted from these clinical sequencing projects can be used to contribute to variant classification. It is possible that some VUSs could have a Signature 3 profile that is sufficiently robust to support classification as likely benign (class 2) or likely pathogenic (class 4), even in the absence of other contributing data. In addition, the contribution of deleterious alterations in other genes, such as *PALB2* and *RAD51C,* to HR defects and hence Signature 3, could lead to the design of therapeutic trials based on the findings described herein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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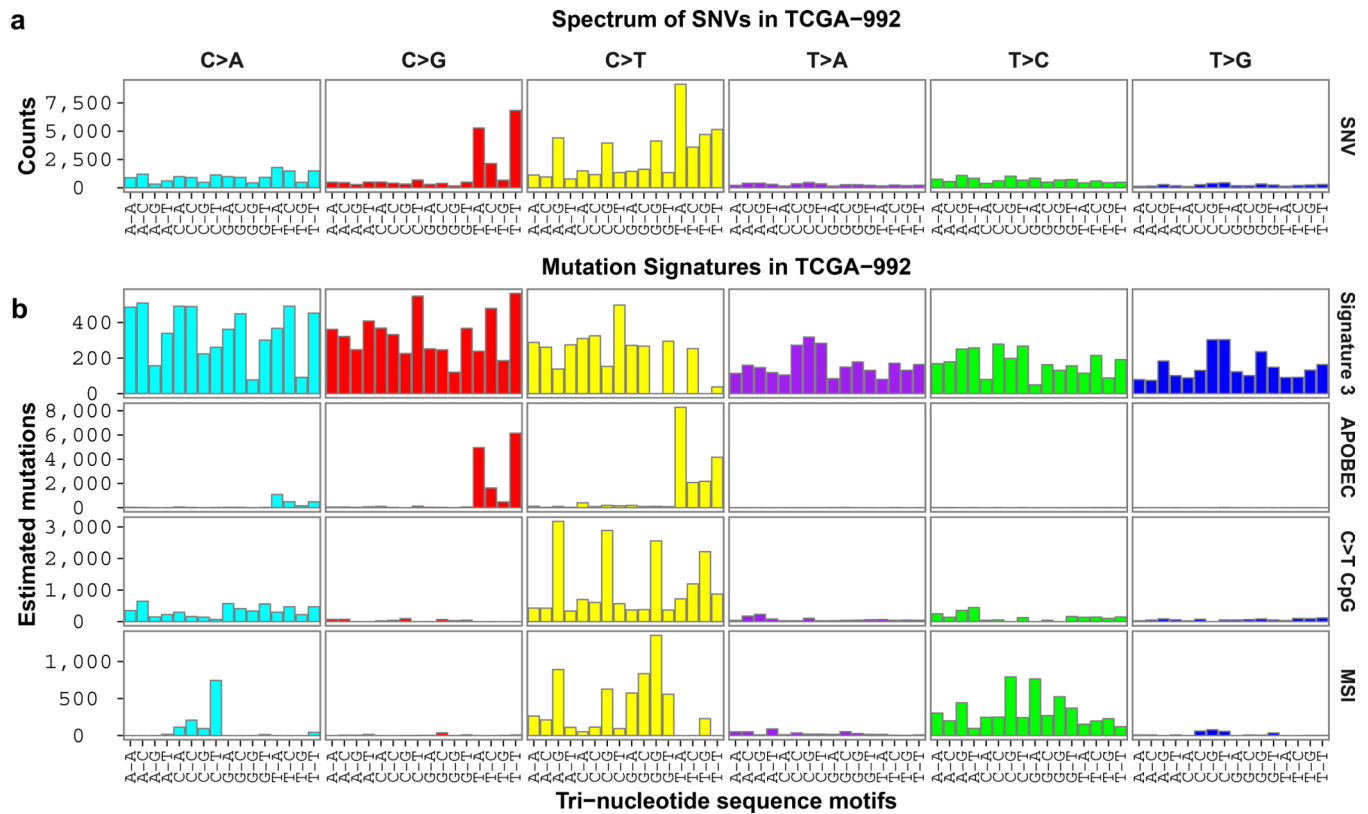
Figure 1: Characterization of four distinct mutational signatures in breast cancer.
A Bayesian non-negative matrix factorization approach (Methods) was employed to decompose the overall mutational spectrum across a cohort of 992 breast cancer samples into distinct mutational signatures ("SNVs" – top row). Each color represents one of the six potential base substitutions, with each substitution further stratified by the adjacent 5' and 3' flanking nucleotides. The pattern of each signature was matched against a database of known signatures and their etiologies (Signature 3, C>T_CpG, APOBEC, MSI). A signature characterized by C>G transversions and C>T transitions at TC[A/T] motifs (in which the altered cytosine is flanked by a 5' thymine and a 3' adenine or thymine) is likely attributable to mutagenesis via endogenous APOBEC machinery ("APOBEC"; COSMIC signatures 2 and 13). A second signature primarily consisting of C>T transitions at CpG dinucleotides has been described among all tumor types and is thought to arise from spontaneous 5-methyl-cytosine deamination ("C>T CpG"; COSMIC signature 1). The third signature resembles previously characterized signatures identified in samples known to have microsatellite instability (MSI) ("MSI"; COSMIC signature 6). A recurrent signature in the cohort closely approximates Signature 3, and is characterized by mutations in all 96 nucleotide contexts, with an increased rate among C:G basepairs as compared to A:T ("Signature 3").
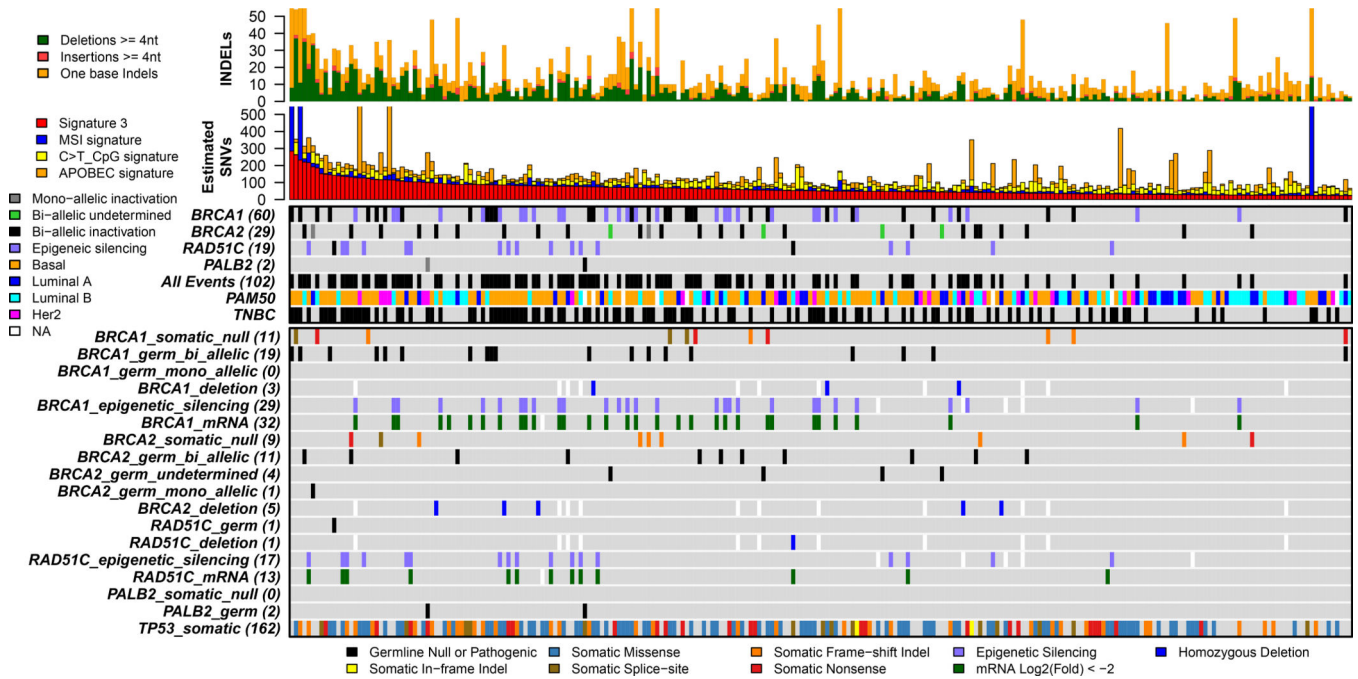
Figure 2: Overall mutation rates, mutational signature contributions, and clinicopathologic features per patient as sorted by descending Signature-3 activity.

Each column represents a tumor sample. Overall indel burden is shown in the top panel with insertion or deletion designated by color. The second panel demonstrates absolute mutation burden per sample, with the estimated contribution of each of the four mutational signatures designated by color (samples are arranged in descending order of Signature 3 activity). The third panel shows sample-level annotations with regard to lesions in known homologous recombination (HR) pathway genes, along with PAM50 intrinsic subtype data, and triple negative status by immunohistochemistry. The bottom panel exhibits genetic lesions by type in select HR genes with mutation type indicated by color. Shown are the first 250 samples by Signature 3 activity; see Supplementary Figure 3 for the rest 742 samples.
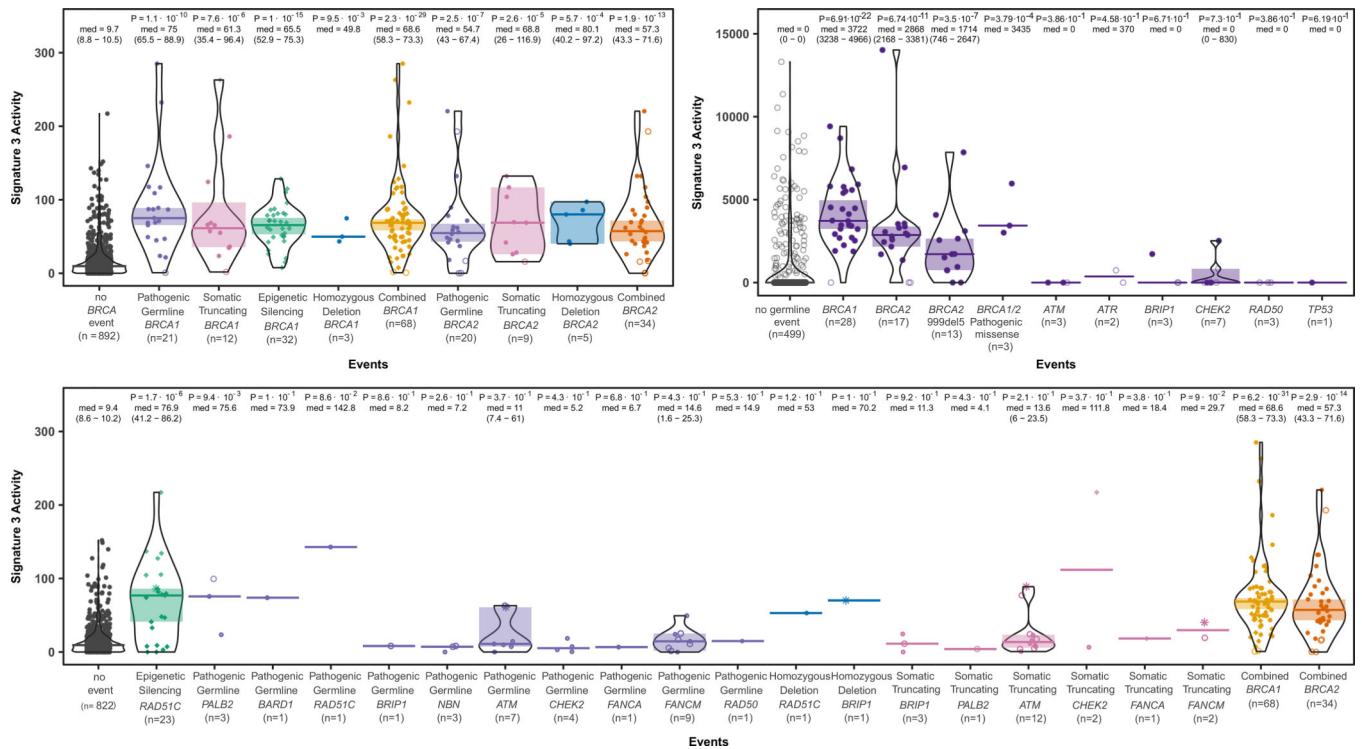
Figure 3: Signature 3 activity in tumors with somatic, germline and epigenetic alterations in HR-pathway genes.

(A) Signature 3 activity is elevated in tumors with somatic, germline and epigenetic alterations in *BRCA1/2.* Tumors were stratified by germline, somatic or epigenetic events in *BRCA1/2* and plotted by number of mutations attributable to Signature 3. Pathogenic germline events included those described by ClinVar, in addition to frameshift and nonsense variants. The median signature count among each group was tested against samples lacking any discernible *BRCA1/2* alterations (denoted as "no *BRCA* event") with two-sided Wilcoxon rank-sum test results as indicated. Open circles represent samples with mono-allelic lesions (i.e. without evidence of biallelic inactivation). Samples with epigenetic silencing are indicated by diamonds. Two samples which harbored a somatic and a germline truncating event in BRCA1/2 and are included in both somatic and germline categories (B) Signature 3 activity in patients with germline alterations as previously denoted[9]. (C) Signature 3 activity is associated with somatic, germline and epigenetic alterations in HR components beyond *BRCA1/2.* Signature 3 activity was compared between samples lacking any of the noted alterations ("no event") versus those with any genetic or epigenetic event in the HR pathway. Samples with an event in *BRCA1/2* were noted by an asterisk. Comparison of Signature 3 activity was conducted with two-sided Wilcoxon rank-sum results noted by group
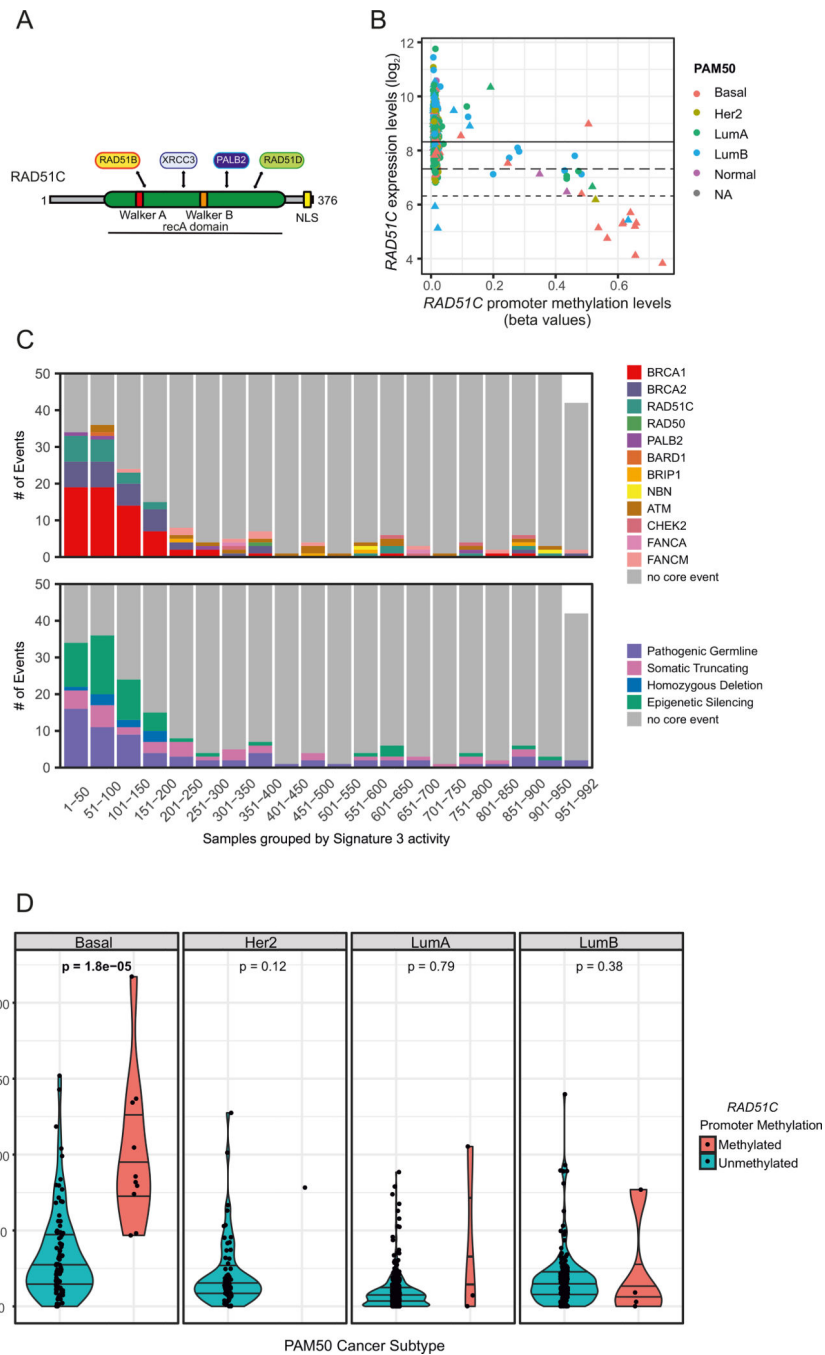
Figure 4: Association of promoter methylation of *RAD51C* with elevated Signature 3 in basal-like tumors.
(A) Schematic representation of RAD51C architecture and its known interacting proteins that promote HR. **(B)** Scatter plot of RAD51C expression as compared to promoter methylation levels (methylation values higher than 0.2 were considered for epigenetic silencing). Samples with high Signature 3 activity (top quartile) are denoted by triangles **(C)** Elevated Signature 3 activity is associated with alterations among multiple components of the HR-pathway and a diversity of alteration types. Samples were ranked by the number of

Signature 3 associated mutations and plotted in descending order in bins of 50. The top panels show alterations among various HR-pathway genes, while the bottom panel shows the types of alterations in these genes, demonstrating enrichment of events among the samples with the highest Signature 3 activity. **(D)** Signature 3 activity was significantly associated with RAD51C promoter methylation among basal-like breast cancers, but not among other intrinsic subtypes.
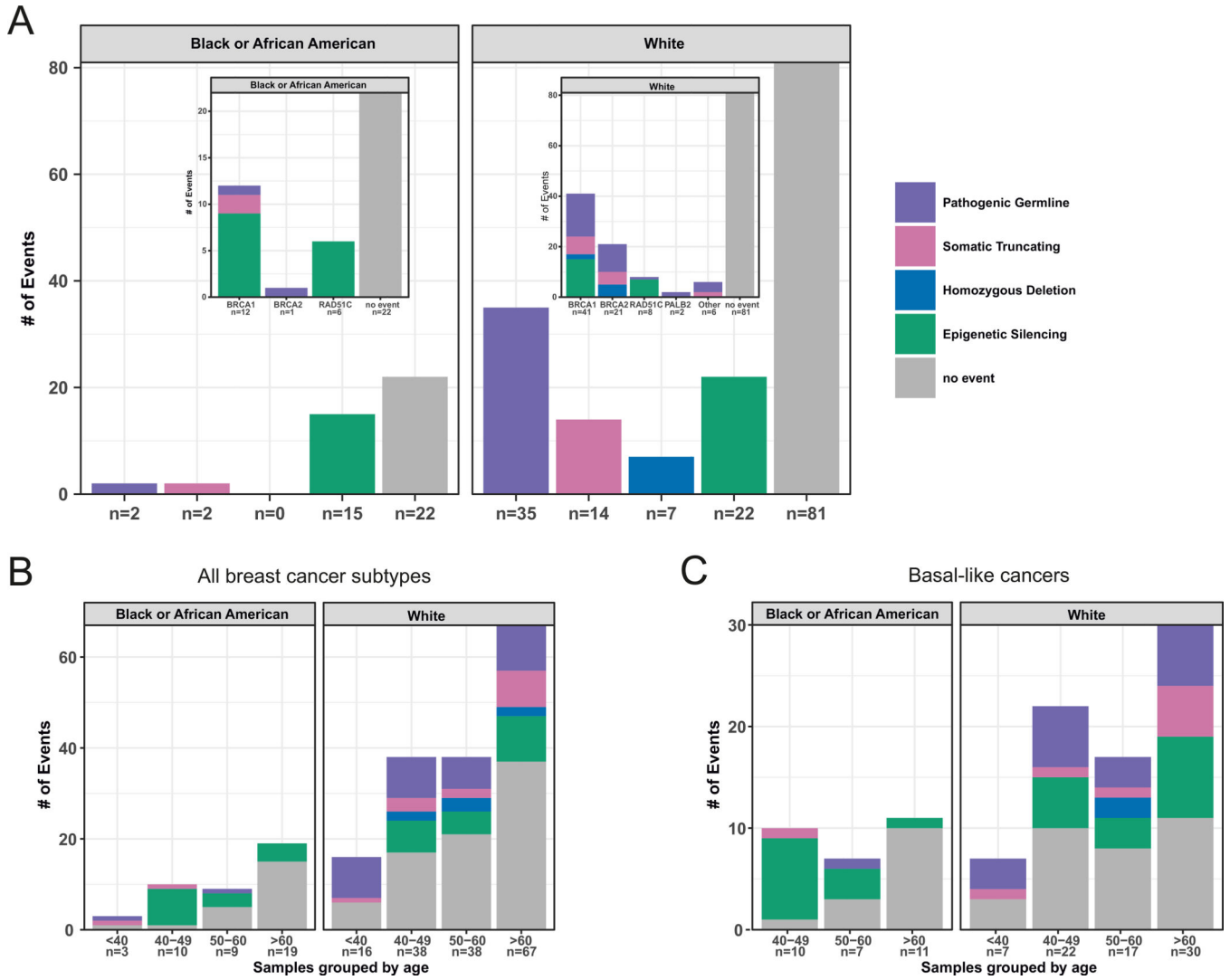
Figure 5: Analysis of genetic and epigenetic events in the 250 samples with highest Signature 3 levels, broken by largest racial subgroups in our cohort (white and African American).
(A) Distribution of various types of alterations among patients stratified by racial groups show an enrichment of promoter methylation in African American patients. The inset shows the distribution of various types of alterations in individual genes. The enrichment of promoter methylation among African American patients is most pronounced in women aged 40–49, both when looking at all breast cancer subtypes (B) and only basal-like cancers (C).
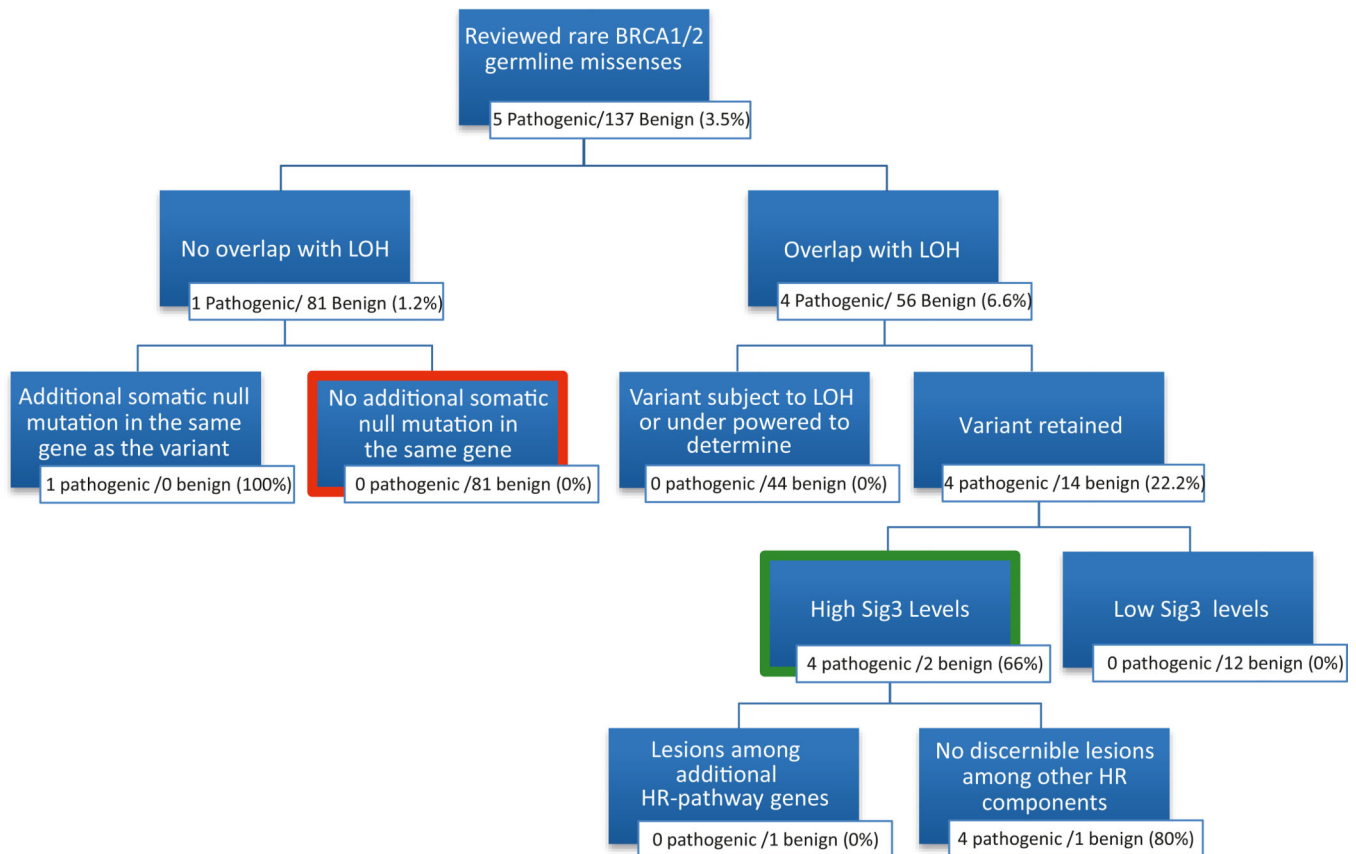
Figure 6: A framework for enhancing the classification of BRCA1/2 germline missense variants using signature 3 and biallelic inactivation.

Beginning with all rare *BRCA1/2* missense germline variants (n=137; 3.5% of randomly selected variants in TCGA breast cancer dataset are pathogenic), the group was dichotomized by presence of LoH at the *BRCA1/2* locus. Those without LOH (n = 82) included only one sample with a pathogenic allele (i.e. 1.2% probability of pathogenicity), although this sample also harbored an additional variant with the potential for biallelic inactivation (indicated by red frame). Conversely, among those with LOH (n= 60; 6.6% of variants are pathogenic), samples were further sorted by allelic imbalance (22.2% of variants are pathogenic), elevated Signature 3 activity (66.6% probability of pathogenicity, indicated by green frame) and the absence of lesions among other HR-pathway components
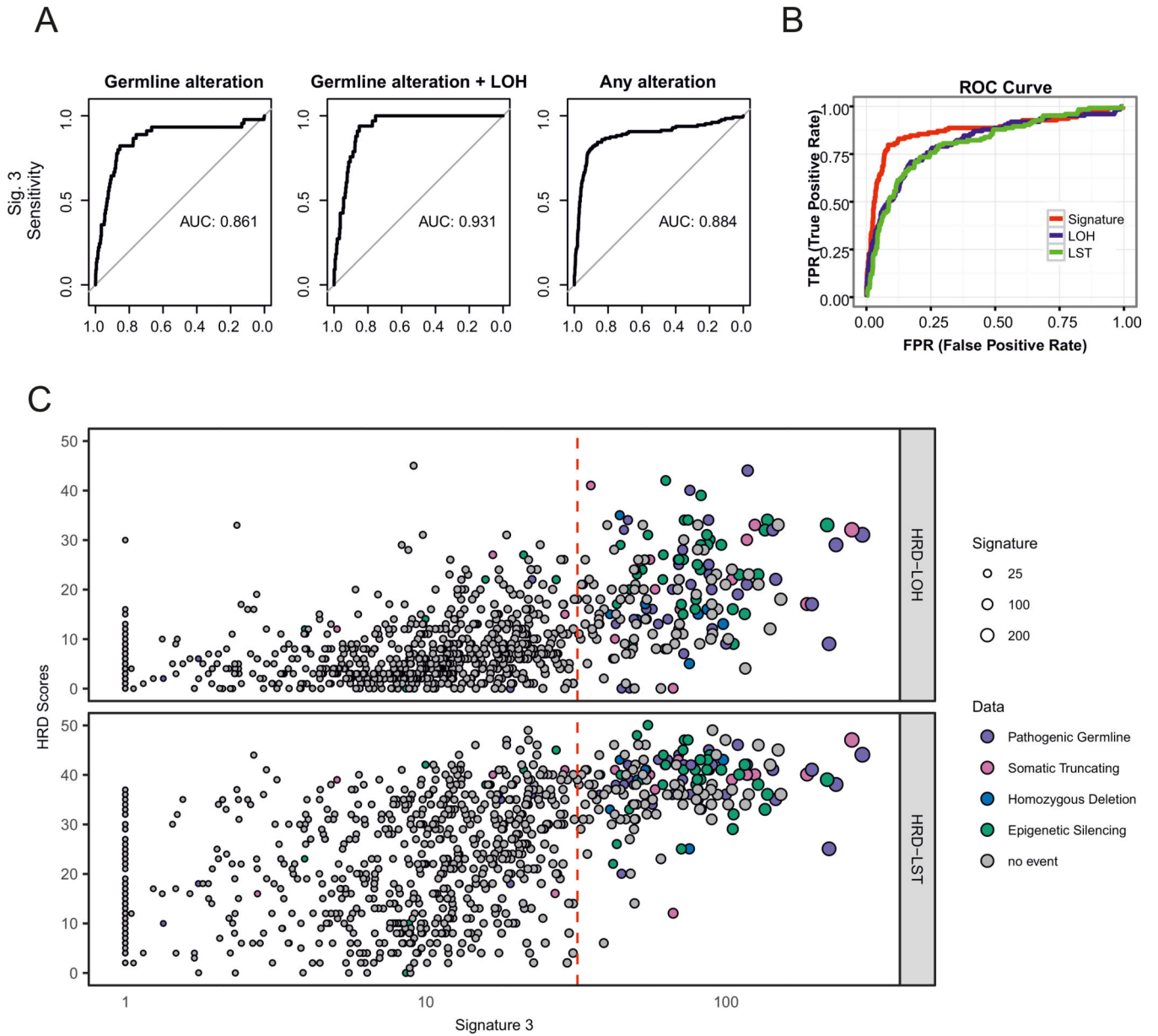
Figure 7: Prediction accuracy of Signature 3 relative to established rearrangement-based HRD scores

(A) The three panels exhibit receiver operating characteristic (ROC) curves for predicted alterations among *BRCA1*, *BRCA2*, *RAD51C*, and *PALB2* using Signature 3 levels. The panels show analyses based on (i) all pathogenic germline mutations (ii) biallelic pathogenic germline mutations and (iii) pathogenic germline, loss of function somatic, or epigenetic alterations. **(B)** ROC curves comparing Signature 3 performance to LOH and LST scores for identifying samples with the HR-associated lesions denoted above. **(C)** Scatter plot exhibiting a comparison of Signature 3 levels versus HRD-LOH (top panel) and HRD-LST scores (bottom panel).