Monomorphic germline pathogenic variants in the Partner And Localizer of BRCA2 (PALB2) gene predispose to a high-risk of breast cancer development, consistent with the role of PALB2 in homologous recombination (HR) DNA repair. Here, we sought to define the repertoire of somatic genetic alterations in PALB2-associated breast cancers (BCs), and whether PALB2-associated BCs display bi-allelic inactivation of PALB2 and/or genomic features of HR-deficiency (HRD). Twenty-four breast cancer patients with pathogenic PALB2 germline mutations were analyzed by whole-exome sequencing (WES, n = 16) or targeted capture massively parallel sequencing (410 cancer genes, n = 8). Somatic genetic alterations, loss of heterozygosity (LOH) of the PALB2 wild-type allele, large-scale state transitions (LSTs) and mutational signatures were defined. PALB2-associated BCs were found to be heterogeneous at the genetic level, with PIK3CA (29%), PALB2 (21%), TP53 (21%), and NOTCH3 (17%) being the genes most frequently affected by somatic mutations. Bi-allelic PALB2 inactivation was found in 16 of the 24 cases (67%), either through LOH (n = 11) or second somatic mutations (n = 5) of the wild-type allele. High LST scores were found in all 12 PALB2-associated BCs with bi-allelic PALB2 inactivation sequenced by WES, of which eight displayed the HRD-related mutational signature 3. In addition, bi-allelic inactivation of PALB2 was significantly associated with high LST scores. Our findings suggest that the identification of bi-allelic PALB2 inactivation in PALB2-associated BCs is required for the personalization of HR-directed therapies, such as platinum salts and/or PARP inhibitors, as the vast majority of PALB2-associated BCs without PALB2 bi-allelic inactivation lack genomic features of HRD.

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been shown to be sensitive to platinum-based chemotherapy and poly (ADP-ribose) polymerase (PARP) inhibitors; hence, therapies targeting HR deficiency (HRD) may benefit breast cancer patients with PALB2 germline mutations.

Although PALB2 constitutes a tumor suppressor gene, there is controversy as to whether it follows the Knudson two-hit model. A recent study revealed that ten of 15 PALB2-associated breast cancers harbored bi-allelic PALB2 inactivation through somatic loss of heterozygosity (LOH) of the PALB2 wild-type allele (n = 6) or somatic PALB2 mutations (n = 4). PALB2 promoter hypermethylation has been reported in tumors from sporadic and BRCA1/2 mutation-negative familial breast and ovarian cancers; however, it appears to be vanishingly rare in tumors from PALB2 germline mutation carriers. Germine to the understanding of the biology of PALB2-associated breast cancers and to the identification of optimal therapeutic approaches for patients with PALB2 germline mutations is to ascertain the mechanisms that contribute to bi-allelic PALB2 inactivation, and to define whether PALB2-associated breast cancers without bi-allelic inactivation lack genomic features consistent with HRD (e.g., large-scale state transitions (LSTs) and mutational signatures). Importantly, Lee et al. based on a targeted capture sequencing analysis of 487 genes, reported that, with one exception, PALB2-associated breast cancers that retained the PALB2 wild-type allele displayed HRD scores consistent with those of tumors harboring PALB2 bi-allelic inactivation.

Here we sought to characterize the repertoire of somatic genetic alterations of breast cancers from pathogenic PALB2 germline mutation carriers using a combination of whole-exome and targeted massively parallel sequencing to de-convolve germline mutation carriers using a combination of whole-exome and targeted massively parallel sequencing to de-convolve germline mutation carriers to that of breast cancers and to the identification of optimal therapeutic approaches for patients with PALB2 germline mutations is to ascertain the mechanisms that contribute to bi-allelic PALB2 inactivation, and to define whether PALB2-associated breast cancers without bi-allelic inactivation lack genomic features consistent with HRD (e.g., large-scale state transitions (LSTs) and mutational signatures). Importantly, Lee et al. based on a targeted capture sequencing analysis of 487 genes, reported that, with one exception, PALB2-associated breast cancers that retained the PALB2 wild-type allele displayed HRD scores consistent with those of tumors harboring PALB2 bi-allelic inactivation.

RESULTS

Clinicopathologic features of PALB2-associated breast cancers

Twenty-four invasive breast cancers from carriers of fourteen distinct pathogenic PALB2 germline mutations were included in this study. Fourteen cases were subjected to WES and WES sequencing data from two cases were retrieved from TCGA (n = 16; median depth of tumor 112 x (range 33 – 289 x) and normal 129 x (range 46 x – 247 x) samples). In addition, 8 cases were analyzed by targeted capture massively parallel sequencing using the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) sequencing assay (median depth of tumor 232 x (range 73 x – 904 x), and normal 545 x (range 172 x – 1452 x) samples; Table 1, Supplementary Table 1). All samples included in this study were derived from formalin-fixed paraffin-embedded (FFPE) material. Sample quality was evaluated and was found to be appropriate for the analyses conducted (Supplementary Table 2). All but one PALB2 germline mutations were bona fide loss-of-function (frame-shift or truncating) mutations; one case carried a missense substitution (L35P), which we have previously demonstrated to be pathogenic.

The median age at breast cancer diagnosis was 49 years (range 29–82 years), and the median tumor size was 1.6 cm (range 0.15–6 cm; Table 1). All PALB2-associated breast cancers were invasive ductal carcinomas of no special type, and one, 12, and eleven cases were of histologic grades 1, 2, and 3, respectively (Table 1). Eighteen cases (75%) were ER-positive/HER2-negative (ER+/-HER2–), five (21%) were ER-negative/HER2-negative (ER–/HER2–), and one (4%) was ER-positive/HER2-positive (ER+/-HER2+; Table 1). Whilst the distribution of PALB2-associated breast cancers into ER/HER2 clinical subgroups was found to be comparable to that of sporadic and BRCA2 breast cancers, it differed from that of BRCA1 breast cancers, which are preferentially of triple-negative phenotype (approximately 70%–85%).

Repertoire of somatic genetic alterations in PALB2-associated breast cancers

Somatic mutation analysis of the WES data of 16 PALB2-associated breast cancers revealed a median of 113.5 (range 59–269) somatic mutations per case, of which 82.5 (range 37–195) were non-synonymous. The eight PALB2-associated breast cancers analyzed by MSK-IMPACT displayed a median of 3 (range 0–5) somatic mutations per case, of which 2 (range 0–4) were non-synonymous (Table 1, Supplementary Table 3). Selected somatic mutations (n = 17) were validated by Sanger sequencing (Supplementary Fig. 1).

Of the 410 cancer genes included in MSK-IMPACT, recurrently mutated genes found in the 24 PALB2-associated breast cancers included PIK3CA (n = 7, 29%), PALB2 (n = 5, 21%), TP53 (n = 5, 21%), NOTCH3 (n = 4, 17%), KMT2A (n = 3, 17%) and ARID1A (n = 3, 17%; Fig. 1, Supplementary Table 3). Six PIK3CA missense mutations affected hotspot residues, including H1047R (n = 3), E545K (n = 1), N345K (n = 1) and C420R (n = 1), and four were predicted to be clonal (Supplementary Fig. 2, Supplementary Table 3). All but one somatic TP53 mutation, all coupled with loss of heterozygosity (LOH) of the TP53 wild-type allele, were predicted to be clonal (Supplementary Fig. 2, Supplementary Table 3). Additional recurrently mutated genes detected in the 16 PALB2-associated breast cancers profiled by WES included CTNN2A, TMPRSS13, KRTAP4–11, LAMAS, KALRN, and COL22A1 (all, n = 3; Supplementary Fig. 3, Supplementary Table 3).

Copy number (CN) analysis revealed recurrent gains of 1q, 8q, 16p, 17q, and 20q, and losses of 1p, 4p, 8p, 11p, and 17p in the 24 PALB2-associated breast cancers analyzed (Fig. 2). Although the majority (n = 18) of cases were ER+/-HER2–, concurrent 1q gains and 16q losses, the hallmark features of luminal breast cancers, were concurrently found in four cases. Loci recurrently amplified included those mapping to 8q21.3 (encompassing the locus of NBN, n = 5) and 8q24.21 (encompassing the locus of MYC, n = 5; Fig. 2).

Bi-allelic PALB2 inactivation

Bi-allelic PALB2 inactivation was found in 16 of the 24 PALB2-associated breast cancers (67%; Table 1, Fig. 1). In eleven cases, the second hit was in the form of LOH of the PALB2 wild-type allele, whereas in five tumors, it was in the form of an inactivating (i.e., truncating or frameshift) somatic PALB2 mutation. Fifteen of the 16 somatic genetic events leading to bi-allelic inactivation of the PALB2 wild-type allele were predicted to be clonal (Fig. 1, Supplementary Table 3), suggesting that bi-allelic PALB2 inactivation and subsequent complete loss-of-function of PALB2 may constitute an early somatic event in the development of a subset of PALB2-associated breast cancers.

PALB2-associated breast cancers with bi-allelic inactivation display genomic features consistent with HRD

We and others have demonstrated that bi-allelic inactivation but not mono-allelic alterations of HR-related genes are associated with genomic features consistent with HRD. Hence, we sought to define whether LST scores and dominant mutational signature 3 would be associated with bi-allelic PALB2 inactivation. LST scores and mutational signatures were inferred in the 16 PALB2-associated breast cancers analyzed by WES, of which 13 cases...
| Case ID | Age (years) | Tumor size (cm) | Grade | ER | HER2 | PALB2 germline mutation | Germline mutation type | Sequencing type | Somatic mutations (n) | Non-synchronous somatic mutations (n) | PALB2 bi-allelic inactivation | LST score* | Mutational signature |
|---------|-------------|-----------------|-------|----|------|------------------------|------------------------|-----------------|---------------------|--------------------------------|-------------------------------|-----------------|---------------------|
| IDC4    | 47          | 1.9             | 2     | +  | −    | c.3504_3505delCT (His1170Phefs) | Frameshift             | WES             | 109                 | 76                            | LOH                          | High           | Signature 3         |
| IDC51   | 82          | 4               | 3     | +  | −    | c.1037_1041delAAGAA (Lys346Thrfs) | Frameshift             | WES             | 189                 | 131                           | LOH                          | High           | Signature 3         |
| IDC60   | 49          | NA              | 2     | +  | −    | c.424 A>T (Lys142*) | Truncating | WES             | 141                 | 103                           | LOH                          | High           | Signature 3         |
| IDC61   | 53          | NA              | 3     | −  | −    | c.509_510delGA (Arg170fs) | Frameshift             | WES             | 118                 | 86                            | LOH                          | High           | Signature 3         |
| IDC55   | 33          | 1.2             | 3     | +  | −    | c.3116delA (Asn1039lfs) | Frameshift             | WES             | 138                 | 106                           | LOH                          | High           | Signature 3         |
| IDC59   | 49          | 1.1             | 2     | +  | −    | c.2323 C>T (Gln775*) | Truncating | WES             | 92                  | 72                            | LOH                          | High           | Signature 3         |
| IDC8    | 42          | 6               | 2     | +  | −    | c.2323 C>T (Gln775*) | Truncating | WES             | 63                  | 41                            | LOH                          | High           | Unstable            |
| IDC9    | 29          | 1.2             | 2     | +  | −    | c.2521delA (Thr841Glnfs) | Frameshift             | WES             | 250                 | 161                           | LOH                          | High           | Signature 1         |
| IDC15   | 54          | 2               | 3     | +  | −    | c.1592delT (Leu531Cysfs) | Frameshift             | WES             | 215                 | 155                           | LOH                          | High           | Signature 1         |
| IDC35   | 64          | 2.4             | 3     | +  | −    | c.3113 G>A (Trp1038*) | Truncating | IMPACT           | 8                   | 8                             | LOH                          | N/A            | N/A                 |
| IDC33   | 49          | 0.48            | 3     | +  | −    | c.3113 G>A (Trp1038*) | Truncating | IMPACT           | 9                   | 6                             | LOH                          | N/A            | N/A                 |
| IDC53   | 32          | NA              | 2     | +  | −    | c.104 T>C (Leu35Pro) | Missense               | WES             | 269                 | 195                           | p.Gln61*                      | High           | Signature 3         |
| IDC50   | 64          | 2               | 3     | +  | −    | c.1037_1041delAAGAA (Lys346Thrfs) | Frameshift             | WES             | 220                 | 147                           | p.Gln921Argfs                 | High           | Signature 3         |
| IDC24   | 51          | 1.2             | 2     | +  | −    | c.3113 G>A (Trp1038*) | Truncating | WES             | 73                  | 54                            | p.Gln479*                     | High           | Signature 1         |
| IDC62   | 52          | 1.9             | 3     | +  | −    | c.172_175delTTGT (Gln60Argfs) | Frameshift             | IMPACT           | 11                  | 10                            | p.Q822*                      | N/A            | N/A                 |
| IDC52   | 44          | 3               | 2     | +  | −    | c.1783delE (Glu584Lysfs) | Frameshift             | IMPACT           | 7                   | 5                             | p.Tyr79fs                    | N/A            | N/A                 |
| IDC37   | 61          | 0.15            | 2     | +  | −    | c.3113 G>A (Trp1038*) | Truncating | WES             | 99                  | 79                            | No                           | Low            | Signature 3         |
| IDC46   | 38          | NA              | 3     | −  | −    | c.1037_1041delAAGAA (Lys346Thrfs) | Frameshift             | WES             | 107                 | 73                            | No                           | High           | Signature 1         |
| IDC28   | 47          | 0.7             | 2     | +  | −    | c.3113 G>A (Trp1038*) | Truncating | WES             | 109                 | 67                            | No                           | Low            | Signature 1         |
| IDC3    | 49          | 1.6             | 3     | +  | −    | c.3504_3505delCT (His1170Phefs) | Frameshift             | WES             | 59                  | 37                            | No                           | Low            | Unstable            |
| IDC49   | 30          | 1.5             | 1     | +  | −    | c.1059delE (Lys353Asnfs) | Frameshift             | IMPACT           | 5                   | 4                             | No                           | N/A            | N/A                 |
| IDC19   | 38          | NA              | 2     | +  | −    | c.172_175delTTGT (Gln60Argfs) | Frameshift             | IMPACT           | 4                   | 4                             | No                           | N/A            | N/A                 |
| IDC13   | 54          | 1               | 3     | −  | −    | c.1592delE (Leu531Cysfs) | Frameshift             | IMPACT           | 9                   | 6                             | No                           | N/A            | N/A                 |
| IDC63   | 48          | 3.7             | 2     | +  | −    | c.2488delE (Glu830lfs) | Frameshift             | IMPACT           | 3                   | 1                             | No                           | N/A            | N/A                 |

LSTs and mutational signatures could only be defined in tumors subjected to WES

Age: age at diagnosis, ER: estrogen receptor, IMPACT: MSK-integrated mutation profiling of actionable cancer targets, LST: large-scale state transition, N/A: not assessable, WES: whole-exome sequencing

*LST high, ≥15; LST low, <15
were found to display high LST scores (LST\textsuperscript{high}), and nine cases (eight LST\textsuperscript{high} and one LST\textsuperscript{low}) were found to have a dominant mutational signature 3 associated with HRD (i.e., BRCA1/2 signature; Table 1, Figs. 1 and 3a). No significant association between PALB2 germline mutation types and HRD-related genomic features was observed (P > 0.05; Table 1). Bi-allelic PALB2 inactivation was significantly associated with LST\textsuperscript{high} (1/4 vs. 12/12, $P = 0.0071$, Fisher’s exact test; $P = 0.009$.

| PALB2 LOH | LST score | Dominant mutational signature | Sequencing platform |
|-----------|-----------|-------------------------------|---------------------|
| LOH       | High (>15) | Signature 1                   | WES                 |
| No LOH    | Low (<15)  | Signature 3                   | MSK-IMPACT          |
| Clonal LOH| N/A       | Unstable                      | N/A                 |

| Receptor status | Mutation type       | Palb2 LOH | LST score | Dominant mutational signature | Sequencing platform |
|-----------------|---------------------|-----------|-----------|-------------------------------|---------------------|
| Positive        | Hotspot missense    | LOH       | High (>15)| Signature 1                   | WES                 |
| Positive        | Truncating SNV      | No LOH    | Low (<15)| Signature 3                   | MSK-IMPACT          |
| Positive        | Frame-shift indel   | Clonal LOH| N/A       | Unstable                      | N/A                 |

| PALB2 germline mutation types and HRD-related genomic features was observed (P > 0.05; Table 1). Bi-allelic PALB2 inactivation was significantly associated with LST\textsuperscript{high} (1/4 vs. 12/12, $P = 0.0071$, Fisher’s exact test; $P = 0.009$,
Mann–Whitney U test; Fig. 3b), in agreement with the findings that bi-allelic inactivation rather than mono-allelic alterations of HR-related genes is associated with HRD-related genomic features.19,24 All but one (IDC37) cases displaying mutational signature 3 and all but one case (IDC46) displaying high LST scores were found to harbor bi-allelic PALB2 inactivation (Table 1, Fig. 1 and Fig. 3b). Notably, we did not identify pathogenic germline mutations, bi-allelic or mono-allelic somatic mutations or homozygous deletions affecting other HRD-related genes in IDC37 or IDC46. Alternative mechanisms of inactivation of the wild-type allele of PALB2, such as complex PALB2 rearrangements or, less likely PALB2 promoter hypermethylation,25 which are not detectable by WES, or other mechanisms that result in HRD may be operative in IDC37 and IDC46. Four of the 12 PALB2-breast cancers with bi-allelic PALB2 inactivation subjected to WES lacked a dominant signature 3, despite displaying high LST scores (Fig. 1). One could hypothesize that these cases could correspond to sporadic breast cancers arising in PALB2 germline mutation carriers, in which the second PALB2 allele was inactivated later in tumor evolution. Two of the 12 PALB2-associated breast cancers analyzed by WES lacked both evidence of bi-allelic PALB2 inactivation and genomic features of HRD (Table 1, Fig. 1). One could posit that these invasive breast cancers may constitute non-PALB2-related cancers arising in the context of a PALB2 germline mutation.

As an exploratory, hypothesis-generating analysis, we compared the genomic profiles of invasive breast cancers developing in the context of pathogenic PALB2 germline mutations with or without loss of the PALB2 wild-type allele. Among the 16 PALB2-associated breast cancers analyzed by WES, the 12 cases with bi-allelic PALB2 inactivation harbored a numerically higher somatic mutation rate (median 139.5, range 63–269) than the four cases without bi-allelic inactivation (median 103, range 59–109; P = 0.09). Moreover, in PALB2-associated breast cancers analyzed by WES and harboring indels (n = 14), the average deletion length was significantly longer in cases with bi-allelic PALB2 inactivation (n = 11) as compared to those with mono-allelic alterations (n = 3; 7 bp vs. 2.3 bp; P = 0.041; Fig. 3c), a feature associated with HRD.26 We further found a significantly higher number of copy number alterations (CNAs) in the tumors with bi-allelic PALB2 inactivation (n = 12) than in those without (n = 4; P = 0.004; Fig. 3d). These results suggest that PALB2-associated breast cancers with bi-allelic inactivation, display higher levels of genetic instability, which may potentially be associated with the early onset of HRD in their development.

Lastly, upon combining the PALB2-associated breast cancers reported by Lee et al.12 with the cases analyzed here, we observed that 67% (26/39) of PALB2-associated breast cancers harbored bi-allelic PALB2 inactivation (Supplementary Table 4). Consistently, bi-allelic PALB2 inactivation was significantly associated with a high LST score, whilst no significant association was observed between bi-allelic PALB2 inactivation and clinicopathologic characteristics (P > 0.05; Supplementary Table 4). PALB2-associated breast cancers with bi-allelic inactivation display higher mutation burden and HRD-associated features more frequently than sporadic breast cancers

As an exploratory, hypothesis-generating analysis we investigated whether PALB2-associated breast cancers would differ from non-BRCA1/2/PALB2-associated breast cancers from TCGA.19 Given that none of the PALB2-associated breast cancers included here was of ER+/HER2− phenotype, ER+/HER2+ non-BRCA1/2/PALB2-associated breast cancers from TCGA were excluded, and the remaining 683 ER−/HER2− and ER+ (including ER+/HER2+ and ER+/HER2−) breast cancers were employed for the analyses. The 16 PALB2-associated breast cancers analyzed by WES were found to harbor a higher number of somatic mutations (median 111.5, range 59–269) than the 683 ER−/HER2− and ER+ non-BRCA1/2/PALB2-associated breast cancers (median 51, range 2–666; P < 0.002, Mann–Whitney U test), difference that remained significant upon 1:3 bootstrap resampling (P = 0.002, see Methods). Given that the majority of the PALB2-associated breast cancers were ER+/HER2−, we restricted the comparison of mutation burden to the 12 ER+/HER2− PALB2-associated breast cancers sequenced by WES (median of somatic mutations 125, range 63–269) and the 441 ER+/HER2+ non-BRCA1/2/PALB2-associated breast cancers (median somatic mutations 42, range 2–666), and the difference remained significant (P < 0.0001, Mann–Whitney U test; P = 0.0002, bootstrapping-corrected). As expected, the 12 PALB2-associated breast cancers with bi-allelic PALB2 inactivation analyzed by WES (ten ER+/HER2− and two ER−/HER2−) harbored a significantly higher number of somatic mutations (median 139.5, range 63–269) than the 568 ER−/HER2− and ER+/HER2− (median somatic mutations 50, range 2–666; P = 0.0001, Mann–Whitney U test; P = 0.005, bootstrapping-corrected). It should be noted that no significant differences in the number of somatic mutations were found between the four PALB2-associated breast cancers analyzed by WES lacking bi-allelic PALB2 inactivation (one ER−/HER2− and three ER+; median 103, range 59–109) and the 683 ER−/HER2− and ER+ non-BRCA1/2/PALB2-associated breast cancers (median 51, range 2–666; P > 0.05, Mann–Whitney U test and bootstrapping-corrected).

A comparison of the frequencies of somatic mutations affecting the 410 cancer genes between PALB2 and non-BRCA1/2/PALB2-associated breast cancers revealed that PALB2, NOTCH3, KMT2A, BRIP1, DNM1L3A, FGFR4, GNAQ, and CD79A (all P < 0.05; Fisher’s exact test) were more frequently mutated in the 24 PALB2-associated breast cancers than in the 683 ER−/HER2− and ER+ non-BRCA1/2/PALB2-associated breast cancers (Fig. 4a), however only PALB2 and NOTCH3 remained significantly differently mutated between the two groups after bootstrap resampling (P < 0.01; Supplementary Table S). No significant differences in the frequency of PIK3CA and TP53 mutations, the two genes most frequently mutated in breast cancer,19 were detected between the 24 PALB2 and the 683 ER−/HER2− and ER+ non-BRCA1/2/PALB2-associated breast cancers (Fig. 4a). Upon restriction of the comparison to the 18 ER+/HER2− PALB2-associated breast cancers and the 441 ER+/HER2− non-BRCA1/2/PALB2-associated

Fig. 1 Non-synonymous somatic mutations in PALB2-associated breast cancers. Heatmap depicting the somatic genetic alterations identified in the 24 PALB2-associated breast cancers analyzed by whole-exome (n = 16) or targeted MSK-IMPACT (n = 8) massively parallel sequencing. Somatic mutations affecting the 410 cancer genes present in MSK-IMPACT, in decreasing overall mutational frequency observed in PALB2-associated breast cancers are plotted. Cases are shown in columns, and genes in rows. Estrogen receptor (ER) and HER2 status, PALB2 germline mutation type, presence of a second somatic PALB2 mutation or loss of heterozygosity (LOH) of the PALB2 wild-type allele, large-scale state transition (LST) score, dominant mutational signature and sequencing platform are indicated in the legend. Note that mutational signatures and LST scores could not be assessed in tumors subjected to MSK-IMPACT sequencing due to the limited number of mutations present. Clonal somatic PALB2 mutations or clonal LOH of the PALB2 wild-type allele are indicated by yellow boxes. Somatic mutations are color-coded according to the legend, and LOH of the wild-type allele of mutated genes other than PALB2 is represented by a diagonal bar. Indel small insertion/deletion; LOH loss of heterozygosity, LST large-scale state transition, N/A not assessable, SNV single nucleotide variant, WES whole-exome sequencing.
breast cancers, PALB2, NOTCH3, ARID1A, DNMT3A, BRIP1, FGFR4, CD79A, and GNAQ (all \( P < 0.05 \); Fisher’s exact test) were significantly more frequently mutated in the PALB2-associated breast cancers (Fig. 4b), but similarly only PALB2 and NOTCH3 remained significantly different after bootstrapping resampling (\( P < 0.01 \), Fisher’s exact test and bootstrapping-corrected; Supplementary Table 5).

Differences in the patterns of CNAs were observed between PALB2-associated breast cancers and non-BRCA1/2/PALB2-associated breast cancers. The 24 PALB2-associated breast cancers harbored gains of 16p and losses of 13p and 16q less frequently than the 683 \( +/\text{HER2}− \) and \( −/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers (\( P < 0.05 \), Fisher’s exact test and bootstrapping-corrected; Supplementary Fig. 4a). When restricting the comparison to the 18 \( +/\text{HER2}− \) PALB2-associated breast cancers and the 441 \( −/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers, the differences were less overt, with more frequent 16p gains and 16q losses in the non-BRCA1/2/PALB2-associated breast cancers (\( P < 0.05 \), Fisher’s exact test and bootstrapping-corrected; Supplementary Fig. 4b). Fewer differences were detected in the comparisons between the eight PALB2-associated breast cancers with mono-allelic inactivation (two \( +/\text{HER2}− \) and four \( -/\text{HER2}− \)) and the 683 \( +/\text{HER2}− \) and \( −/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers (Supplementary Fig. 4c). No significant difference in the frequency of amplifications and homozygous deletions was observed in any of the comparisons when the bootstrap resampling analysis was performed (\( P > 0.05 \); Supplementary Fig. 4d–f).

As part of the exploratory analysis, we investigated whether PALB2-associated breast cancers would differ from non-BRCA1/2/PALB2-associated breast cancers in regard to the frequencies of genomic features indicative of HRD. The 12 PALB2-associated breast cancers with bi-allelic inactivation (two \( −/\text{HER2}− \) and ten \( +/\text{HER2}− \) analyzed by WES) were found to display significantly higher LST scores than the 526 \( +/\text{HER2}− \) and \( −/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers for which LST scores could be determined (\( P < 0.0001 \), Mann–Whitney \( U \) test; \( P = 0.0001 \), bootstrapping-corrected; Fig. 4c). By contrast, the four PALB2-associated breast cancers with mono-allelic inactivation (one \( −/\text{HER2}− \) and three \( +/\text{HER2}− \), sequenced by WES) displayed comparable LST scores to the 634 \( −/\text{HER2}− \) and \( +/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers for which LST scores could be defined (\( P > 0.05 \), Mann–Whitney \( U \) test and bootstrapping-corrected; Fig. 4c). Likewise, the proportion of cases displaying a mutational signature 3 was significantly higher in the 12 PALB2-associated breast cancers with bi-allelic inactivation sequenced by WES than in the 491 \( +/\text{HER2}− \) and \( −/\text{HER2}− \) non-BRCA1/2/PALB2-associated breast cancers for which mutational signatures could be inferred (67% vs. 17%; \( P = 0.0002 \), Fisher’s exact test; \( P = 0.02 \), bootstrapping-corrected; Fig. 4c). These results suggest that PALB2-associated breast cancers with bi-allelic inactivation are more often HR-deficient than non-BRCA1/
2/PALB2-associated breast cancers despite displaying a similar prevalence of ER-positive luminal breast cancers, and that PALB2-associated breast cancers without bi-allelic inactivation appear to resemble non-BRCA1/2/PALB2-associated breast cancers. PALB2-associated breast cancers with bi-allelic inactivation display similarities with BRCA1- and BRCA2-associated breast cancers with bi-allelic inactivation of BRCA1/2. Finally, we sought to define whether PALB2-associated breast cancers with bi-allelic inactivation would differ from breast cancers arising in BRCA1 and BRCA2 pathogenic germline mutation carriers with bi-allelic inactivation of BRCA1 and BRCA2, respectively. The 12 PALB2-associated breast cancers analyzed by WES were found to harbor a number of somatic mutations (median 139.5, range 63–269) comparable to that of 17 BRCA1-associated breast cancers with bi-allelic inactivation from TCGA (median 143, range 54–1223; \( P > 0.05 \), Mann–Whitney U test), and higher than that of the 16 BRCA2-associated breast cancers with bi-allelic inactivation from TCGA (median 74.5, range 38–209; \( P = 0.006 \), Mann–Whitney U test). In regards to the repertoire of somatic mutations, PALB2 mutations were significantly more frequent in the 16 PALB2-associated breast cancers with bi-allelic inactivation ( \( n = 5, 31\% \)) than in the 17 BRCA1- ( \( n = 0 \)) and 16 BRCA2-associated ( \( n = 0 \)) breast cancers with bi-allelic inactivation from TCGA ( \( P = 0.02 \) and \( P = 0.04 \), respectively, Mann–Whitney U test; Fig. 5a, b, Supplementary Table 5). In addition, a higher frequency of TP53 mutations was found in the 17 BRCA1-
associated breast cancers ($n = 15, 88\%) than in the 16 PALB2-associated breast cancers with bi-allelic inactivation ($n = 3, 19\%$; $p < 0.0001$, Fisher's exact test; Fig. 5a).

CN analysis revealed that the 17 BRCA1-associated breast cancers with bi-allelic inactivation had higher frequencies of gains of 3q and 6p and losses of 17q, among other differences ($P < 0.05$, Fisher's exact test; Supplementary Fig. 5a), as compared to the 16 PALB2-associated breast cancers with bi-allelic inactivation. In contrast, the CN profiles of the 16 BRCA2 breast cancers with bi-allelic inactivation were more similar to those of the PALB2-associated breast cancers, albeit more frequently harboring losses of 13q and 22q, among other differences ($P < 0.05$; Supplementary Fig. 5b). No significant difference in the frequency of amplifications and homozygous deletions was found between the 16 PALB2-associated breast cancers and the 17 BRCA1-associated and 16 BRCA2-associated breast cancers with bi-allelic inactivation of the respective wild-type allele (Supplementary Fig. 5c, d).

The LST scores of the 12 breast cancers with bi-allelic PALB2 inactivation analyzed by WES were comparable to those of the breast cancers with bi-allelic BRCA1 inactivation ($n = 17$) and bi-allelic BRCA2 inactivation from TCGA ($n = 16$; $P > 0.05$, Mann–Whitney U test; Fig. 5c), whereas the proportion of the PALB2-associated breast cancers with bi-allelic inactivation displaying signature 3 (67%, 8/12) was not statistically significantly different from that of the BRCA1-associated breast cancers with bi-allelic inactivation (82%, 14/17; $P = 0.4$, Fisher's exact test) and BRCA2-associated breast cancers with bi-allelic inactivation from TCGA (63%, 10/16; $P = 1$, Fisher's exact test; Fig. 5c). Consistent with these findings, LST score, NtAI score, $^{26}$ which assesses telomeric allelic imbalance, and the Myriad score, $^{26}$ which is the unweighted sum of LOH, telomeric allelic imbalance and LSTs, were higher in breast cancers with PALB2 ($n = 12$) and BRCA1/2 ($n = 33$) biallelic inactivation, compared to those with PALB2 ($n = 4$) and BRCA1/2 ($n = 8$) monoallelic inactivation, respectively (Supplementary Figs. 6a–6c). Moreover, LST score, mutational signature 3, NtAI score and Myriad score detected bi-allelic inactivation of PALB2 and of BRCA1/BRCA2 in PALB2-associated breast cancers and in BRCA1/2-associated breast cancers, respectively, with comparable accuracy (Supplementary Figs. 6d–6g). Taken together, our results suggest that PALB2-associated breast cancers with bi-allelic inactivation are similar to breast cancers with BRCA1 and BRCA2–bi-allelic inactivation in terms of genetic instability and genomic features indicative of HRD.

**DISCUSSION**

Here we demonstrate that PALB2-associated breast cancers constitute a heterogeneous group of tumors at the genetic level and can be stratified according to the bi-allelic inactivation of the PALB2 wild-type allele. PALB2-associated breast cancers display a high mutation burden and a limited number of genes recurrently affected by pathogenic somatic mutations, including PIK3CA, TP53, NOTCH3, and PALB2 itself. Loss of the PALB2 wild-type allele in PALB2-associated breast cancers occurred in the form of PALB2 pathogenic somatic mutations in five (21%) cases, whereas LOH of the wild-type allele of PALB2 was detected in 11 (46%) cases. Second somatic mutations in BRCA1/2 have been reported as the underlying cause of bi-allelic inactivation in tumors from BRCA1/2 germline mutations carriers. $^{27}$ It should be noted, however, that somatic mutations resulting in the inactivation of the wild-type allele of BRCA1 or BRCA2 in BRCA1– or BRCA2-associated breast cancers, respectively, $^{15,24,28}$ appear to be less frequent than somatic PALB2 mutations in the context of PALB2-associated breast cancers. In the study by Maxwell et al. $^{28}$ bi-allelic BRCA1 inactivation was due to a BRCA1 somatic mutation in only one case (1.1%) out of 93 BRCA1-associated breast and ovarian tumors. Similarly, out of 67 BRCA2-associated tumors with bi-allelic BRCA2 inactivation, in only one case was a somatic mutation (1.5%). In contrast, PALB2 somatic mutations as a mechanism of bi-allelic inactivation were significantly more frequent in the PALB2-associated breast cancers from this series (31%; 5/16; $P = 0.00006$, Fisher's exact test).

Consistent with the findings of Lee et al., $^{12}$ our study demonstrates that PALB2 follows the Knudson two-hit model, given that in a large proportion of PALB2-associated breast cancers, a second hit in the form of a somatic PALB2 mutation or LOH of the wild-type allele of PALB2 was detected. Contrary to that study, $^{12}$ in which PALB2-associated breast cancers with either mono-allelic or bi-allelic PALB2 alterations were found to display genomic features of HRD, based on targeted massively parallel sequencing of 487 genes, our WES analysis of 16 PALB2-associated breast cancers revealed that tumors with PALB2 bi-allelic alterations displayed significantly higher LST scores and average deletion lengths than PALB2-associated breast cancers with mono-allelic PALB2 alterations. In addition, only one out of the four PALB2-associated breast cancers with mono-allelic PALB2 alterations displayed a dominant mutational signature 3, whereas eight out of 12 PALB2-associated breast cancers with bi-allelic PALB2 inactivation harbored a dominant mutational signature 3. Our WES findings are consistent with the pan-cancer WES analysis performed by Riaz et al., $^{13}$ whereby HR-related genes with bi-allelic inactivation but not those with mono-allelic alterations were
found to display genomic features of HRD, and the analyses performed by Polak et al.,\textsuperscript{24} where bi-allelic, but not mono-allelic, alterations affecting *BRCA1*, *BRCA2*, and *PALB2* were found to be associated with HRD in breast cancers. Conversely, 8/24 *PALB2*-associated breast cancers included in this study lacked bi-allelic *PALB2* inactivation and 2/16 *PALB2*-associated breast cancers sequenced by WES lacked both bi-allelic *PALB2* inactivation and genomic features of HRD. In this context, one could posit that this subset of *PALB2*-associated breast cancers may retain competent HR repair of DNA double-strand breaks and would unlikely benefit from HRD-directed therapies. Interestingly, the proportion of *PALB2*-associated breast cancers displaying mono-allelic *PALB2* A. Li et al. \textsuperscript{9} Published in partnership with the Breast Cancer Research Foundation npj Breast Cancer (2019) 23
inactivation was comparable to the one of BRCA1-associated and BRCA2-associated breast cancers from TCGA harboring BRCA1 or BRCA2 mono-allelic inactivation, respectively. Although PALB2 mono-allelic inactivation is not associated with genomic features of HRD, its role in tumorigenesis is yet to be determined.

In agreement with previous studies showing that most breast cancers with HRD features are underpinned by bi-allelic inactivation of HR-related genes,\textsuperscript{15,29} we identified the genetic basis of HRD in 12 out of 14 (86%) PALB2-associated breast cancers with genomic features of HRD. It should be noted that of the PALB2-associated breast cancers with mono-allelic PALB2 alterations studied here, one displayed a high LST score and another one harbored a dominant mutational signature 3. This observation suggests that other mechanisms of HRD may be operative in these tumors. First, no bi-allelic inactivation of another DNA repair related gene was detected in these cancers. Second, albeit PALB2 gene promoter methylation was reported in two of eight inherited breast cancers and four of 60 sporadic breast cancers,\textsuperscript{11} this phenomenon appears to be vanishingly rare in PALB2-associated breast cancers with mono-allelic PALB2 alterations.\textsuperscript{12} Further analyses are warranted to define whether other mechanisms of inactivation of the wild-type allele of PALB2 may play a role in PALB2-associated breast cancers with mono-allelic PALB2 alterations but with genomic features of HRD.

Our study has important limitations. First, as a result of the rarity of PALB2-associated breast cancers, the small sample size may have limited the detection of significant differences in the exploratory analyses comparing PALB2-associated breast cancers with non-BRCA1/2/PALB2-associated breast cancers from TCGA. Importantly, however, these analyses revealed that PALB2-associated breast cancers with bi-allelic inactivation differ from non-BRCA1/2/PALB2-associated breast cancers but are similar to BRCA1 and BRCA2 breast cancers with bi-allelic inactivation. Second, genomic features of HRD were investigated here based on WES; although our approach for the detection of genomic features of HRD is more robust than that based on targeted massively parallel sequencing,\textsuperscript{12} whole-genome sequencing analysis still remains the optimal approach. Therefore, the proportion of PALB2-associated breast cancers with HRD may be even higher than that reported here and in previous analyses.\textsuperscript{12}

Despite these limitations, our data demonstrate that PALB2-associated breast cancers harbor complex and heterogeneous genomes. Notably, PALB2 bi-allelic inactivation is present in a large proportion of PALB2-associated breast cancers, and the mechanisms leading to this include both LOH of the wild-type allele or pathogenic somatic mutations affecting PALB2. Importantly, the 12 PALB2-associated breast cancers with PALB2 bi-allelic inactivation displayed genomic features consistent with HRD, and shared similarities in terms of genetic instability and genomic features of HRD with BRCA1-associated and BRCA2-associated breast cancers with bi-allelic inactivation. Two of the 16 PALB2-associated breast cancers subjected to WES, however, lacked both bi-allelic inactivation of PALB2 and genomic features of HRD. Hence, we posit that molecular assays to identify bi-allelic inactivation of PALB2 and/or genomic features of HRD may aid in the selection of patients likely to benefit from HRD-directed therapies, including platinum-based chemotherapy and/or PARP inhibitors.

**METHODS**

**Cases**

We included 24 invasive breast cancers from women with pathogenic PALB2 germline mutations. This study was approved by Memorial Sloan Kettering Cancer Center’s institutional review board (IRB) and by the local ethics committees/IRBs of the authors’ institutions. Written informed consents were obtained as required by the protocols approved by the IRBs/local ethics committees of the respective authors’ institutions. This study is in compliance with the Declaration of Helsinki. For 22 breast cancers, tissue samples were retrieved from the McGill University (Canada, \textit{n} = 6), Cancer Research Malaysia/University Malaya (Malaysia, \textit{n} = 5), the Kathleen Cunningham Foundation Consortium for research into Familial Breast Cancer (KConFab, Australia, \textit{n} = 5), Memorial Sloan Kettering Cancer Center (MSKCC; New York, USA, \textit{n} = 3), University of Eastern Finland (Finland, \textit{n} = 2) and Charles University (Czech Republic, \textit{n} = 1). Hematoxylin and eosin-stained tissue sections of the 22 breast cancers were reviewed by three pathologists (FCG, FP, and JSR-F). The genomics data of BRCA1/2/PALB2-P-associated breast cancers and non-BRCA1/2/PALB2-associated breast cancers from TCGA, the 34 cases lacking LST scores but displaying mutational signatures are not shown three of these cases display signature 3. *Of the 491 ER−/HER2− breast cancers, the 29 cases lacking LST scores but displaying mutational signatures are not shown, three of these cases display signature 3. **Of the comparisons of LST scores are shown using Fisher’s exact tests. N/A signatures not assessable, LST large-scale state transition.

**Immunohistochemistry and fluorescence in situ hybridization (FISH)**

ER and HER2 status were assessed by immunohistochemistry following American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines.\textsuperscript{30} In addition, HER2 amplification was assessed in selected cases by fluorescence in situ hybridization (FISH) using PathVysion (Abbott) and/or HER2 IQFISH pharmDx (Dako), following the ASCO/CAP guidelines.\textsuperscript{31,32}

**DNA extraction**

Eight-micrometer-thick sections from representative FFPE blocks were microdissected with a sterile needle under a stereomicroscope (Olympus) to ensure >80% of tumor cells. Genomic DNA was extracted from tumor and matched normal blood or saliva samples using the DNeasy Blood and Tissue Kit (Qiagen), and quantified using the Qubit Fluorometer (Life Technologies).

Massively parallel sequencing and bioinformatics analysis

DNA of tumor and matched normal samples was subjected to WES (\textit{n} = 14) or MSK-IMPACT\textsuperscript{30} (\textit{n} = 8), which targets all exons and selected introns of...
Cases are shown in columns, and genes in rows. Multi-Fisher’s exact test comparisons of mutational frequencies of the recurrently mutated genes were performed between a the 16 PALB2-associated breast cancers with bi-allelic PALB2 alterations and 17 BRCA1 breast cancers bi-allelic BRCA1 alterations, and b the 16 PALB2-associated breast cancers with bi-allelic PALB2 alterations and 16 BRCA2 breast cancers with bi-allelic BRCA2 alterations. P-value of each comparison is shown on the right side of the heatmap, with statistically significant P-values in bold. Indel, small insertion/deletion; SNV, single nucleotide variant. c Boxplots showing the large-scale state transition (LST) scores of the 12 PALB2-associated breast cancers with bi-allelic PALB2 alterations, 17 BRCA1 and 16 BRCA2 breast cancers with bi-allelic BRCA1 and BRCA2 alterations, respectively. The median value of the LST scores, and the 75th and 25th percentiles are displayed at the top and bottom of the boxes, respectively. Each dot corresponds to the LST score and/or mutational signature of one case. Mutational signatures are color-coded according to the legend. P-values of the comparisons of LST scores are shown using Fisher’s exact tests. N/A signatures not assessable, LST large-scale state transition

The dominant mutational signature in each case was defined based on the consensus of at least two of the three methods.

Sanger sequencing validation
Selected somatic mutations with MAFs >10%, including mutations affecting PIK3CA (n = 7), PALB2 (n = 4), TP53 (n = 4), and NOTCH3 (n = 2), were validated by Sanger sequencing (primer sequences in Supplementary Table 3). PCR amplification of genomic DNA and analyses were performed in duplicate.

Comparisons with breast cancers from TCGA
The mutation burden, mutation frequencies, CNAs and genomic features indicative of HRD of the PALB2-associated breast cancers were compared to those of non-BRCA1/2/PALB2-associated breast cancers with matched ER and HER2 status (n = 683), and to those of BRCA1 (n = 17) and BRCA2 (n = 16) breast cancers with bi-allelic inactivation from TCGA (Supplementary Methods).

Statistical analysis
Comparisons of the number of somatic mutations and LST scores, gene-level copy number states and mutational signatures between PALB2-associated breast cancers and non-BRCA1/2/PALB2-associated, BRCA1-associated and BRCA2-associated breast cancers were performed using the Mann–Whitney U test and Fisher’s exact test, respectively. To account for differences in sample sizes, a bootstrap resampling analysis was performed (Supplementary Methods).
DATA AVAILABILITY

WES sequencing data (supporting Figs. 1–5, Table 1, Supplementary Figs. 2–6 and supplementary tables 1–5) and MSK-IMPACT sequencing data (supporting Figs. 1, 2, 4 and 5, Table 1, supplementary Figs. 2, 4 and 5 and supplementary tables 1–5) generated during this study, can be accessed from cbioPortal (https://identifiers.org/cbioportal/cbio_brca_mscl_li_2019). TCGA Breast Cancer sequencing data (supporting Figs. 4 and 5, supplementary Figs. 4–6 and supplementary table 5) used in this study, can be accessed from cbioPortal (https://identifiers.org/cbioportal:brca_tgca_pan_can_atlas_2018) or from the related publication https://doi.org/10.1016/j.cell.2018.02.060. Additional data supporting supplementary table 4 can be accessed from table 2 and supplementary table 1 of the related publication: https://doi.org/10.1038/s41523-019-0115-9. The data generated and analyzed during this study are described in the following data record: https://doi.org/10.6084/m9.78138912.

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AUTHOR CONTRIBUTIONS

B.W., S.H.T, M.T., W.D.F. and J.S.R.-F. conceived the study. kConFab Investigators, ZR, T.N.-D., P.P., C.T., T.T., K.R., S.E.B., A.M., R.W., M.I., P.R., B.X., P.S.N., L.-M.L. and M.S. provided samples. F.C.G., R.B. and J.S.R.-F. reviewed the cases. P.B., J.Y.L., P.S., D.N.B., S. S.K.L. and R.K. performed the bioinformatics analysis. A.L., F.C.G., P.B., J.Y.L., P.S., D.N.B., F.P., S.S.K.L., R.K., B.R., B.B., S.P., H.Y.W., J.R.L., R.G.-M., L.C., L.N., M.E.R. analyzed and interpreted the data. A.L., F.C.G., F.P., B.W. and J.S.R.-F. wrote the first paper, which was reviewed by all coauthors.

ADDITIONAL INFORMATION

Supplementary information accompanies the paper on the npj Breast Cancer website (https://doi.org/10.1038/s41558-019-0115-9).

Competing interests: M.E.R. reports consultancy fees from McKesson and AstraZeneca, and uncompensated consulting/ advisory activities with Merck and Pfizer. J.S.R.-F. reports personal/ consultancy fees from VolitionRx, Page.AI, Goldman Sachs, Grail, Ventana Medical Systems, Invicro, and Genentech, outside the scope of the submitted work. All remaining authors declare no competing interests.

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