Heterozygous PALB2 c.1592delT mutation channels DNA double-strand break repair into error-prone pathways in breast cancer patients

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Hereditary heterozygous mutations in a variety of DNA double-strand break (DSB) repair genes have been associated with increased breast cancer risk. In the Finnish population, PALB2 (partner and localizer of BRCA2) represents a major susceptibility gene for female breast cancer, and so far, only one mutation has been described, c.1592delT, which leads to a sixfold increased disease risk. PALB2 is thought to participate in homologous recombination (HR). However, the effect of the Finnish founder mutation on DSB repair has not been investigated. In the current study, we used a panel of lymphoblastoid cell lines (LCLs) derived from seven heterozygous female PALB2 c.1592delT mutation carriers with variable health status and six wild-type matched controls. The results of our DSB repair analysis showed that the PALB2 mutation causes specific changes in pathway usage, namely increases in error-prone single-strand annealing (SSA) and microhomology-mediated end-joining (MMEJ) compared with wild-type LCLs. These data indicated haploinsufficiency regarding the suppression of error-prone DSB repair in PALB2 mutation carriers. To the contrary, neither reduced HR activities, nor impaired RAD51 filament assembly, nor sensitization to PARP inhibition were consistently observed. Expression of truncated mutant versus wild-type PALB2 verified a causal role of PALB2 c.1592delT in the shift to error-prone repair. Discrimination between healthy and malignancy-presenting PALB2 mutation carriers revealed a pathway shift particularly in the breast cancer patients, suggesting interaction of PALB2 c.1592delT with additional genomic lesions. Interestingly, the studied PALB2 mutation was associated with 53BP1 accumulation in the healthy mutation carriers but not the patients, and 53BP1 was limiting for error-prone MMEJ in patients but not in healthy carriers. Our study identified a rise in error-prone DSB repair as a potential threat to genomic integrity in heterozygous PALB2 mutation carriers. The used phenotypic marker system has the capacity to capture dysfunction caused by polygenic mechanisms and therefore offers new strategies of cancer risk prediction.

Received 19 February 2015; revised 29 September 2015; accepted 15 October 2015; published online 7 December 2015

INTRODUCTION

Hereditary mutations in a variety of genes involved in double-strand break (DSB) repair genes are associated with an increased risk of developing female breast cancer. However, only a fraction of approximately 30% of this hereditary cancer risk is explained by currently known high-penetration susceptibility genes. A large portion of the remaining predisposition to breast cancer may be explained by a polygenic model involving a combination of multiple genomic risk factors, including the effect of low-penetrance susceptibility alleles. These include polymorphisms in genes involved in several metabolic pathways, signal transduction and DNA repair.

In the Finnish population, PALB2 was reported to represent the most notable breast cancer susceptibility gene along with the previously identified BRCA1 and BRCA2 genes. Interestingly, there is only one single aberration in PALB2 described in this population so far, namely the relatively common c.1592delT protein truncation founder mutation. In females, this mutation leads to a sixfold increase of the breast cancer risk and occurs in 0.2% of the general population. Several investigations have now shown that monoallelic mutations in PALB2 increase the risk of developing female breast cancer, whereas biallelic mutations cause Fanconi anemia (FA) of subtype FA-N. A hallmark of FA patient cells is defectiveness in interstrand crosslink repair, a feature that is being made use of in diagnostic patient classification. DNA strand breaks, particularly DSBs, are the most lethal genomic lesions. In mammalian cells, DSBs are repaired by two main pathways: non-homologous end-joining (NHEJ) and homologous recombination (HR). DSBs generated during crosslink repair are normally subject to HR repair. HR starts with processing of the DNA end, thereby forming single-stranded DNA (ssDNA) 3’ ends, which are quickly covered by the human ssDNA-binding protein RPA. DNA end processing involves the MRE11-RAD50-Nibrin and BRCA1-CtIP complexes. PALB2 acts as the mediator between BRCA1 and BRCA2 via independent interactions at its N- and C-terminus, respectively. The C-terminus...
additionally binds RADS1C, another breast/ovarian cancer susceptibility as well as FA gene product, thus forming a HR complex also containing XRCC3 and RAD51.22,23 BRCA1 may concentrate PALB2 at DNA damage sites in chromatin,70,21 where PALB2 recruits and permits stable localization of BRCA2 to these focal intranuclear sites.19,24,25 BRCA2 promotes the assembly of RAD51 into nucleoprotein filaments thereby replacing RPA.26,27 RAD51 nucleoprotein filaments invade the sister chromatid in search for homology. This represents the central step of HR. It either starts repair synthesis during the unilateral exchange process called synthesis-dependent strand annealing or formation of double Holliday Junctions, which are later resolved in a crossover or non-crossover manner during canonical HR.77 PALB2 has also been reported to bind to ssDNA where it interacts with RAD51 to stimulate strand invasion.28 Importantly, similar HR-related processes that require PALB2, BRCA2 and RAD51 have also a central role in the reactivation of stalled replication forks.29–33 Altogether, PALB2 has been described to exhibit indirect and direct functions in RAD51-dependent HR, which is considered to be the most accurate DSB repair pathway. High fidelity repair by HR requires perfect homologies between the donor and the acceptor sequence, which is why it takes place in the S/G2-phase, when identical sister chromatids are available.34 For comparison, NHEJ, in particular the alternative microhomology-mediated end-joining (MMEJ) sub-pathway, and an alternative RAD51-independent homologous DSB repair pathway, namely single-strand annealing (SSA), are error-prone DSB repair mechanisms that are not limited to a particular cell cycle phase.35,36 NHEJ can result in the loss or gain of nucleotides and hence in the disruption of genomic integrity. Similarly, SSA starts with DNA end processing, however, followed by DNA annealing between genomic repeat sequences with extensive homologies. SSA always leads to the loss of the intervening DNA sequences and, therefore, is considered mutagenic.35

In a previous study, we observed enhanced error-prone DSB repair activities, namely MMEJ and SSA, in lymphoblastoid cell lines (LCLs) from BRCA1 and BRCA2 mutation carriers, respectively.36 As PALB2 was discovered to physically connect BRCA1 and BRCA2,19–21 we mechanistically dissected DSB repair activities in LCLs derived from female individuals heterozygous for the Finnish PALB2 c.1592delT founder mutation. The breast cancer risks associated with PALB2 mutations have been reported to synergize with additional genetic factors.37,38 To better understand the impact of polygenic interactions with the Finnish founder mutation on cancer risk-associated DSB repair dysfunction, we compared cohorts with and without PALB2 c.1592delT mutation and with and without breast cancer. The data presented in this study demonstrate that mutated PALB2 derepresses SSA and MMEJ, a phenotype which very likely cooperates with additional changes in carrier individuals with cancer, such as deregulation of S3BP1 levels associated with the lack of protection against excessive end processing.

RESULTS

Individuals heterozygous for the Finnish PALB2 founder mutation show specific changes in DSB repair pathway usage

To determine whether and how DSB repair is altered in individuals heterozygous for the Finnish c.1592delT PALB2 founder mutation, we introduced different EGFP-based reporter constructs into LCLs from PALB2 mutation carriers and healthy individuals with wild-type PALB232,39 (Supplementary Table 1). Our systematic analysis of pathway-specific DSB repair activities addressed NHEJ, MMEJ, SSA, SSA+HR and HR (Figures 1a and b and representative FACS plots in Supplementary Figure 1), where PALB2 wild-type LCL BR-0968 served as internal reference. As summarized in Table 1, we observed statistically significant increases in NHEJ (1.5- to 3.4-fold), MMEJ (1.8- to 9.6-fold), SSA (1.6- to 7.9-fold) and/or SSA+HR (1.8- to 10.9-fold) in five LCLs from PALB2 mutation carriers, encompassing individuals without (healthy) and with breast cancer (brca). However, in LCL BR-0970 from a breast cancer patient undergoing cytostatic therapy (brca+therapy), no such increases were observed, rather a reduction such as for SSA+HR (to 70%). As the only other exception, significant downregulation of SSA (to 60%) was found in LCL BR-0724 (brca). Regarding HR, we obtained no statistically significant changes in PALB2-mutated LCLs, except for BR-0970 (brca+therapy) displaying HR downregulation to 20%. Notably, BR-0970 cells from the breast cancer patient undergoing therapy were the only ones that did not match any of the DSB repair changes observed with the other LCLs from PALB2 mutation carriers and were therefore excluded from any cohort analysis in this work. Accordingly, augmentation of NHEJ, MMEJ, SSA and SSA+HR was detected in 60% of PALB2-mutated LCLs. In conclusion, DSB repair analysis of individual LCLs revealed a shift to error-prone DSB repair pathways in PALB2 mutation carriers with and without breast cancer before therapy.

To take the possibility into account that the internal reference BR-0968 may not perfectly represent the wild-type phenotype, we performed cohort analysis for the informative pathways NHEJ, MMEJ, SSA and SSA+HR. Thus, we calculated mean values of specific DSB repair activities from six PALB2-mutated LCLs (excluding BR-0970 from the patient undergoing therapy) and six matched LCLs with wild-type PALB2 (for individual analysis of LCLs see Table 1). When comparing the two different genotypes, no statistically significant change in NHEJ was observed. However, the mutation carriers showed a 1.8-fold increase in MMEJ (P = 0.022) and SSA (P = 0.001) and a 1.9-fold increase in SSA+HR (P = 0.002) in relation to the wild-type cohort (Figure 2a, left panel). Moreover, plotting MMEJ changes in PALB2-mutated LCLs (Table 1) against the respective PALB2 protein levels according to our previous study32 revealed a statistically significant inverse correlation (P = 0.0167; Spearman r, two-tailed). When subdividing the group of PALB2-mutated LCLs, we noticed a statistically significant 2.0-fold increase in SSA (P = 0.0034) and a 2.0-fold increase in SSA+HR (P = 0.003) compared with the wild-type LCLs. Under the transient assay conditions applied to patient cells, analysis of SSA+HR was previously found to primarily reflect SSA activities, so that the SSA+HR data further supported increased usage of this pathway.40,41 In summary, cohort analysis confirmed MMEJ and SSA upregulation in PALB2 c.1592delT-mutation carriers versus 15% of wild-type controls. When subdividing the group of mutation carriers into healthy and breast cancer-affected individuals, we found a difference only for NHEJ, namely a rise in those with breast cancer. The same subgroup also showed elevated SSA versus the wild-type cohort (not healthy mutation carriers versus the wild-type cohort). Surprisingly, overexpression of wild-type PALB2 did not affect SSA+HR in carriers or wild-type control LCLs (Figure 2b). Instead overexpression of the truncated PALB2 encoded by PALB2 c.1592delT caused a 1.6- and 1.5-fold increase in SSA+HR (P < 0.0001).

Heterozygously mutated PALB2 rarely associates with sensitivity to PARP inhibitor treatment

Following the detection of ssDNA breaks, poly(ADP-ribosyl) transferase (PARP)1–3 activate various DNA repair pathways and stabilize stalled replication forks.42 Resolution of stalled forks requires HR, that is, the pathway PALB2 is thought to have a role in.32 PARP inhibition was shown to eliminate BRCA1- and
PALB2 physically and functionally interacts with BRCA1 and BRCA2.39 Representatively shown is the construct enabling HR between a mutated EGFP–acceptor sequence with an I-SceI recognition site and a donor sequence representing truncated EGFP. A plasmid mixture, containing the DSB repair substrate HR-EGFP/5'EGFP, I-SceI-meganuclease expression plasmid (pCMV-I-SceI), and either filler plasmid pBS or wild-type EGFP expression plasmid were introduced into the LCLs by electroporation followed by cultivation for 48 h. DSB repair was triggered by I-SceI-mediated DSB formation followed by the restoration of EGFP and appearance of green fluorescent cells which were analyzed flow cytometrically. As proof-of-principle, BR-0968 and BR-0967 cells with wild-type and heterozygously mutated PALB2 were transfected according to the protocol and cells harvested 24 h later. Expression of the HA-I-SceI fusion protein was verified by immunoblotting with antibody directed against the HA-tag. Transfection efficiencies were determined for each sample and used to individually normalize DSB repair frequencies thereby excluding potential frequency changes related to transfection, transcription, translation, proliferation and lethality. (b) Constructs for specific DSB repair analysis. Constructs for the assessment of different DSB repair pathways are shown: NHEJ (EJ5SceGFP), MMEJ (EJ-EGFP), SSA (5'EGFP/HR-EGFP), SSA+HR (HR-EGFP/3'EGFP) and HR (HR-EGFP/5'EGFP). The NHEJ substrate contains two I-SceI recognition sites. Mutated EGFP genes, light gray boxes; deleted EGFP sequence, cross; I-SceI recognition site, white triangle; microhomologies, gray triangles; promoter sequence, gray box; spacer sequence, black box.

Figure 1. Assay for comparison of DSB repair pathway usage in individuals with wild-type and heterozygously mutated PALB2. (a) Assay principle for the analysis of DSB repair pathways in LCLs.39 Representatively shown is the construct enabling HR between a mutated EGFP–acceptor sequence with an I-SceI recognition site and a donor sequence representing truncated EGFP. A plasmid mixture, containing the DSB repair substrate HR-EGFP/5'EGFP, I-SceI-meganuclease expression plasmid (pCMV-I-SceI), and either filler plasmid pBS or wild-type EGFP expression plasmid were introduced into the LCLs by electroporation followed by cultivation for 48 h. DSB repair was triggered by I-SceI-mediated DSB formation followed by the restoration of EGFP and appearance of green fluorescent cells which were analyzed flow cytometrically. As proof-of-principle, BR-0968 and BR-0967 cells with wild-type and heterozygously mutated PALB2 were transfected according to the protocol and cells harvested 24 h later. Expression of the HA-I-SceI fusion protein was verified by immunoblotting with antibody directed against the HA-tag. Transfection efficiencies were determined for each sample and used to individually normalize DSB repair frequencies thereby excluding potential frequency changes related to transfection, transcription, translation, proliferation and lethality. (b) Constructs for specific DSB repair analysis. Constructs for the assessment of different DSB repair pathways are shown: NHEJ (EJ5SceGFP), MMEJ (EJ-EGFP), SSA (5'EGFP/HR-EGFP), SSA+HR (HR-EGFP/3'EGFP) and HR (HR-EGFP/5'EGFP). The NHEJ substrate contains two I-SceI recognition sites. Mutated EGFP genes, light gray boxes; deleted EGFP sequence, cross; I-SceI recognition site, white triangle; microhomologies, gray triangles; promoter sequence, gray box; spacer sequence, black box.

BRCA2-mutated tumor cells in preclinical and clinical studies.43 As PALB2 physically and functionally interacts with BRCA1 and BRCA2,19–22 sensitivity to PARP inhibition was assessed in the PALB2-mutated LCLs. For this purpose, LCLs were treated with different concentrations of the PARP inhibitor 1,5-isoquinolinediol and subjected to MTT assay. As a proof-of-principle, we showed that this approach may detect HR dysfunction in a gene dose-dependent manner, because LCL cells with biallelic BRCA2 mutation (GM13023A) showed 3.8-fold higher sensitivity (P = 0.0138) than heterozygously BRCA2-mutated (HA238) cells, that is, highest sensitivity compared with wild-type control cells (TK6) (Table 2). When analyzing PALB2-mutated LCLs, we observed statistically significantly reduced IC50 values for BR-0760 (brca) and BR-0970 (brca-therapy) versus the wild-type reference BR-0968. But none of the other five PALB2-mutated LCLs showed a significant increase of PARP inhibitor sensitivity. IC50 values of five additional LCLs with wild-type PALB2 were also not significantly different from the wild-type reference BR-0968. Consistently, cohort analysis of LCLs from wild-type PALB2 and PALB2-mutated donors with or without breast cancer revealed no differences between the mean IC50 values of the subgroups (Figure 3).

Analysis of DSB repair components reveals accumulation of 53BP1 in healthy PALB2 mutation carriers
To further delineate the molecular defect caused by heterozygous PALB2 mutation in DSB repair, we performed immunofluorescence microscopic analysis of nuclear structures indicative of the accumulation and/or removal of DSBS (53BP1) and the assembly/disassembly of the HR repair machinery (RAD51).41 Depending on the selected genotoxic treatment, distinct DNA repair pathways can be addressed such as canonical NHEJ in the early repair phase after irradiation, HR after treatment with the crosslinker Mitomycin C (MMC).16,18,34

First, we induced DSBs in LCLs by exposure to ionizing radiation (2 Gy γ-ray) and subjected the cells to immunostaining and quantitative immunofluorescence microscopy at different time points post irradiation. Accumulation of 53BP1 0.5–1 h after ionizing radiation was followed by a gradual decrease of foci numbers indicating DSB formation and subsequent repair both in wild-type and PALB2-mutated LCLs (Supplementary Figure 2a). When monitoring RAD51 filament assembly post irradiation, we detected similar kinetics of foci accumulation and decline as with 53BP1 (Supplementary Figure 2b). A statistically significant difference in 53BP1 or RAD51 foci numbers specific for a
PALB2–mutated LCL versus the wild-type reference BR-0968 was only observed in one single case each (Supplementary Figure 1 and further data not shown). To focus on HR, we treated the LCLs with MMC inducing a gradual increase of 53BP1 foci numbers up to 6 h after exposure indicating DSB formation during crosslink processing. MMC treatment did not unveil any difference in 53BP1 accumulation in individual LCLs (Supplementary Figure 2c). RPA coats ssDNA and becomes phosphorylated at the 32 kDa subunit (P-RPA) when accumulating on DNA. P-RPA foci kinetics post ionization radiation did not reveal differences between LCLs from family I members with wild-type or mutated PALB2 (Supplementary Figure 2d). Cohort analysis of LCLs from wild-type and PALB2–mutated donors with or without breast cancer verified the increases in foci numbers for both treatment modalities and time points post treatment (Figure 4). It further demonstrated complete absence of significant differences between the subgroups (wild-type, PALB2–mutated and healthy, PALB2–mutated and breast cancer) regarding 53BP1 and RAD51 foci/nucleus before and after irradiation as well as 53BP1 foci/nucleus before and after MMC treatment. When recalculating foci data regarding the percentage of responding cells (53BP1: ≥5 foci/nucleus, RAD51: ≥2 foci/nucleus), no statistically significant differences were observed except for a small, namely 7% decrease (P = 0.0302) of 53BP1-positive cells 6 h post-MMC in the breast cancer patients with mutated versus wild-type PALB2 controls.

In another approach to unravel the molecular components involved in the DSB repair pathway shift in PALB2–mutated individuals, we used western blotting to quantify the phosphorylated repair components indicating DNA damage and repair intermediates, respectively. The damage marker γH2AX detects different types of lesions including stalled replication forks, which accumulate after MMC treatment. P-RPA represents a marker of DSB end processing. We performed at least two independent immunoblotting experiments for each of the LCLs from Finnish donors, normalized antigen-specific signals with the loading control and the internal wild-type reference BR-0968 each, and compared values for the wild-type cohort versus PALB2–mutated healthy individuals and breast cancer patients (Figure 5, western blots for individual LCLs in Supplementary Figure 3).

Regarding γH2AX, we observed a trend towards decreased signals in untreated LCLs from mutation carriers with breast cancer down to 41% of the level in the wild-type cohort, which was not seen for the healthy mutation carrier LCLs (Figure 5). UponRAD52 knockdown, diminished SSA (and MMEJ, also called C/HMEJ) was observed in PALB2–mutated LCLs (Figure 4, western blots for individual LCLs in Supplementary Figure 3). Compared with the wild-type, RAD52 knockdown in PALB2–mutated breast cancer patients (Figure 5) did not reveal any significant differences between the cohorts. Finally, we investigated endogenous protein levels of key factors implicated in pathway choice decisions. Thus, we examined the important regulator of DSB signaling 53BP1, which promotes NHEJ and antagonizes homologous repair, MRE11, the nuclease component of the DSB recognizing and end processing MRE11-RAD50-Nibrin complex as well as the downstream nuclease CtIP, PARP1, which has been implicated in alternative NHEJ, and RAD51 the central recombinase in HR. 

Interestingly, we observed a 2.3-fold increase of 53BP1 protein levels in the cohort of healthy mutation carriers compared with the wild-type cohort that was lost in the PALB2–mutated breast cancer patients (Figure 5). For comparison, none of the other proteins (MRE11, PARP1, RAD51, CtIP) showed quantitative changes. Suggesting posttranslational stabilization of 53BP1 in PALB2–mutated healthy individuals versus breast cancer patients, we observed no change and a 53BP1 decrease, respectively, following exposure to the protein biosynthesis inhibitor Cycloheximide (Supplementary Figure 4a). Consistent with 53BP1’s proposed role, we found a statistically significant decrease of NHEJ upon 53BP1 knockdown in PALB2 mutation carriers with and without breast cancer (Supplementary Figure 4b). MMEJ was dependent on 53BP1 only in breast cancer patients. For comparison, inactivation/knockdown of the key factors of HR (RAD51) and SSA (RAD52) caused increased and decreased SSA, respectively, in cells from wild-type PALB2 control individuals (Supplementary Figure 5).

Upon RAD52 knockdown, diminished SSA (and MMEJ, also called micro-SSA) was also noticeable in cells from the healthy PALB2 mutation carrier, but not from the breast cancer patient of the same family. Therefore, RAD52-independent mechanisms promote SSA in PALB2–mutated cells from the breast cancer patients.
DISCUSSION

The EMBRACE study demonstrated the dramatic effect of modifier genes on the cancer risk of BRCA2 mutation carriers. These results underscore the need for comprehensive parameters, which describe the combined breast cancer risk of high-penetrance susceptibility genes in the individual’s genetic background. In our previous study, we demonstrated an association between distinct DSB repair activities and breast cancer risk. The heterozygous

![Graphs showing DSB repair frequency for different conditions](image-url)
Finnish PALB2 c.1592delT founder mutation leads on average to a sixfold increase in breast cancer risk.\textsuperscript{9} In our study, we elucidated the DSBR repair phenotype of female PALB2 c.1592delT mutation carriers as compared with wild-type individuals.

The heterozygous PALB2 germline mutation most prominently caused increases in the error-prone activities MMEJ (2- to 10-fold) and even more significantly in SSA/SSA+HR (2- to 11-fold) as determined via analysis of cohorts and comparison of individual carrier LCLs versus wild-type reference. These data indicated a mixed phenotype of PALB2 mutation carrier cells as compared with heterozygously BRCA1-mutated cells, which had correspondingly been characterized by 3- to 16-fold elevated MMEJ and heterozygously BRCA2-mutated cells with threefold increased SSA activities.\textsuperscript{5, 25} This mixed cellular phenotype reflects the physical interactions of PALB2 connecting BRCA1 and BRCA2.\textsuperscript{19–21} As previously observed with heterozygously BRCA1- and BRCA2-mutated cells,\textsuperscript{25} HR was not consistently found to be impaired in heterozygously mutated PALB2 cells. This finding can partially be explained by the fact that HR frequencies were closer to the detection limit, which made HR and a further decrease of this activity more difficult to detect. On the other hand, a fivefold HR decrease was in fact measured in one PALB2-mutated cell type (Table 1). Another explanation could be that alternative HR repair mechanisms engaging RAD52 or involving template switch or break-induced replication, which are not or less sensitive to lack of RAD51 and BRCA2,\textsuperscript{48–50} became prevalent in PALB2 mutation carrier cells. Finally, the possibility remains that the PALB2

| Cell line | Genotype | Health status | IC50\textsuperscript{a} | P-value\textsuperscript{b} |
|-----------|----------|---------------|----------------|------------------|
| TK6       | Wild-type| Healthy       | 76              | –                |
| HA238     | BRCA2 (c.5946delCT/wt) | brca | 45              | 0.0464          |
| GM13023A | Biallelic BRCA2 mutation | Fanconi anemia | 12              | <0.0001          |
| BR-0968   | Wild-type| Healthy       | 39              | –                |
| BR-0778   |          |               | 23              | 0.1300           |
| BR-0781   |          |               | 57              | 0.0556           |
| BR-1016   |          |               | 47              | 0.4853           |
| BR-1017   |          |               | 26              | 0.5245           |
| BR-1023   |          |               | 38              | 0.7369           |
| BR-0954   | PALB2 (c.1592delT/wt) | Healthy | 41              | 0.6937           |
| BR-0967   |          |               | 38              | 0.6241           |
| BR-0724   |          | brca          | 45              | 0.7500           |
| BR-0736   |          |               | 36              | 0.8263           |
| BR-0737   |          |               | 24              | 0.4956           |
| BR-0760   |          |               | 16              | 0.0469           |
| BR-0970   | brca+therapy |              | 6               | 0.0432           |

Abbreviations: brca, breast cancer patient; brca+therapy, breast cancer patient undergoing cytostatic therapy; LCLs, lymphoblastoid cell lines. LCLs were treated with increasing concentrations of 1,5-isoquinolinediol (IQD; 2 μM–2 mM). Cell viability was assessed using MTT assay as described in the legend to Figure 3. IC50 values (μM) were determined from 4 to 18 survival curves and statistical significance calculated using F-test of Log IC50 with software GraphPad Prism version 5.04 (numbers of survival curves: BR-0954 6, BR-0967 4, BR-0724 6, BR-0736 6, BR-0737 8, BR-0760 6, BR-0970 4, BR-0778 10, BR-0781 10, BR-1016 6, BR-1017 6, BR-1023 4 and reference BR-0968 18)). \textsuperscript{b}P-values of differences between IC50 values determined in parallel were calculated for BRCA2-mutated LCLs versus for wild-type LCL TK6 and for PALB2-mutated LCLs versus for wild-type reference LCL BR-0968. *Sensitivity to PARP inhibitor IQD treatment (exposure to 2 μM–2 mM for 6 days) was assessed by calculating IC50 values (μM) from 4 to 18 survival curves by use of the software GraphPad Prism version 5.04. \textsuperscript{b}P-values of differences between IC50 values determined in parallel were calculated for BRCA2-mutated LCLs versus for wild-type LCL TK6 and for PALB2-mutated LCLs versus for wild-type reference LCL BR-0968.

Figure 2. Cohort analysis of DSBR repair pathway usage in wild-type and heterozygously PALB2-mutated individuals with and without breast cancer. (a) DSBR repair as a function of the endogenous PALB2 status. LCLs from six wild-type PALB2 individuals (white columns) and six PALB2 c.1592delT mutation carriers (dark gray columns, left panel), among those two from healthy individuals (light gray columns, right panel) and four from breast cancer patients (dark gray columns, right panel), were transfected with reporter constructs for analysis of NHEJ, MMEJ, SSA and SSA+HR and repair frequencies determined as described in the legend to Figure 1. Frequencies relative to the reference cell line BR-0968 with wild-type PALB2 (100%) were determined in 6–36 measurements (average of absolute values corresponding to 100%: NHEJ: 2.9 × 10\textsuperscript{-3}; MMEJ: 5.2 × 10\textsuperscript{-3}; SSA: 2.3 × 10\textsuperscript{-3}; SSA+HR: 2.4 × 10\textsuperscript{-3}). Data are shown in box plots including mean value (cross) and median (line), 95% confidence interval (CI). Statistical analyses with SPSS were performed using general linear mixed models with mutation status (wild-type or PALB2-mutated, left panel) or mutation and health status (wild-type, PALB2-mutated and healthy, PALB2-mutated and breast cancer; right panel) as fixed factor and date of the experiment as random factor; asterisks indicate a statistically significant difference. (b) DSBR repair as a function of the exogenous PALB2 status. LCLs from five to six wild-type PALB2 individuals (except BR-1023 for wild-type PALB2 expression, white columns) and all six PALB2 c.1592delT mutation carriers not undergoing therapy (gray columns) were transfected with the plasmid mixture for analysis of SSA+HR plus expression plasmid for wild-type PALB2 (wtPALB2), the mutated variant (mutPALB2) or empty vector. DSBR repair frequencies were determined as described in (a). Mean DSBR repair frequencies of each cell line transfected with the empty vector were set to 100%. Data are shown in box plots including mean value (cross) and median (line), 95% CI. Statistically significant differences were calculated by SPSS using Mann–Whitney U test. Asterisks indicate a statistically significant difference. Expression of exogenous PALB2 proteins in LCLs was verified by immunoblotting of lysates with antibody directed against the protein-tag (representatively shown for one wild-type control and one mutation carrier LCL expressing HA-tagged wild-type PALB2; an arrow marks the band specific for HA-tagged PALB2). α-Tubulin was used as the loading control.

Figure 3. Cohort analysis of PARP inhibitor sensitivities. To determine the cell viability after treatment with the PARP inhibitor 1,5-isoquinolinediol (IQD), increasing IQD concentrations (2 μM–2 mM) were applied. Media were replaced with fresh media including the corresponding IQD concentrations every second day. Cell viability was measured using MTT assay. Mean IC50 values (μM) were determined from 4 to 18 survival curves per LCL and statistical significances calculated using F-test of Log IC50 with software GraphPad Prism version 5.04. The mean IC50 values were visualized as columns in three cohorts, encompassing six LCLs with wild-type PALB2 (white columns), two LCLs derived from healthy PALB2 mutation carriers (light gray columns), and four LCLs from PALB2 mutation carriers with breast cancer (dark gray columns). Bars show s.d. (n = 2–6).
c.1592delT/wt) status is associated with haploinsufficiency regarding the suppression of error-prone DSB repair activities but with haplos sufficiency regarding stimulation of RAD51-dependent HR. In agreement with this concept, we monitored normal RAD51 foci kinetics in most of the PALB2 mutation carrier cell lines. Moreover, the accumulation and decline of DSBs as indicated by 53BP1 foci after irradiation or MMC treatment were similar in wild-type and mutated cell types. A clue to haploinsufficiency regarding the suppression of error-prone repair could be that the truncated PALB2 c.1592delT gene product is highly unstable. Upon overexpression, truncated PALB2 may also exert a dominant-negative influence via the N-terminal BRCA1-binding site\textsuperscript{21,32} as suggested from the observed increase in error-prone DSB repair. BRCA1 via its association with CtIP is involved in the pathway choice, as it promotes DSB end resection and therefore homologous repair rather than classical NHEJ\textsuperscript{26}. In normal cells, BRCA1 promotes RAD51-dependent HR via its association with PALB2. Blocking PALB2 but not CtIP interactions will favor RAD51-independent homologous repair like SSA and other unscheduled processes requiring ssDNA overhangs like MMEJ.

Measurements of total NHEJ, encompassing canonical and alternative activities, revealed augmented usage of NHEJ in the majority of PALB2 mutation carrier cell lines, however, also in several wild-type PALB2 LCLs. Previous data indicated the involvement of not only MMEJ but also SSA in generating chromosomal rearrangements that can lead to malignancies\textsuperscript{51}.
Notably, a reduced capacity to reactivate stalled replication forks as well as chromosomal aberrations including complex rearrangements that are mechanistically linked with homologous repair activities have been observed in PALB2 c.1592delT mutation carriers.32 Fully error-prone DSB repair activities (MMEJ, SSA) were increased in all studied PALB2 mutation carrier LCLs except for two from breast cancer patients. One of those two cell lines, namely from the patient undergoing therapy, was particular as it did not exhibit elevated NHEJ. 52 Hence, mutation carriers with breast cancer more frequently used error-prone DSB repair pathways, which are known to induce genomic instability, and therefore are likely to have accelerated malignancy formation. We propose that additional genetic alterations in this group of PALB2 mutation carriers exacerbated the HR defect and usage of error-prone DSB repair pathways and thus breast cancer risk.

The principle of synthetic lethality has been pursued in PARP inhibitor trials targeting HR-defective tumors.43 In line with the proficiency in HR of the heterozygous PALB2 c.1592delT mutation carriers, we found increased sensitivity to the drug only in two PALB2 mutation carrier cell lines derived from breast cancer patients compared with wild-type controls and of NHEJ in PALB2 mutation carriers with breast cancer (dark gray columns). Bars show s.d. (n = 2–6). brca+ther, breast cancer patient undergoing cytostatic therapy.

When performing cohort analysis, we confirmed the increase of MMEJ and SSA in the PALB2 c.1592delT mutation carriers. Subgroup analysis further revealed a rise of SSA in the breast cancer patients compared with wild-type controls and of NHEJ in the breast cancer patients compared with the healthy mutation carriers. Considering that PALB2 is a FA gene,13,14 our finding on NHEJ is in line with earlier reports demonstrating that FA cells exhibit elevated NHEJ.32 Hence, mutation carriers with breast cancer more frequently used error-prone DSB repair pathways, which are known to induce genomic instability, and therefore are likely to have accelerated malignancy formation. We propose that additional genetic alterations in this group of PALB2 mutation carriers exacerbated the HR defect and usage of error-prone DSB repair pathways and thus breast cancer risk.
patients. One possibility to explain PARP inhibitor sensitivity in these two but not the other LCLs could be that additional genetic or epigenetic alterations might have exacerbated the defect.

Of interest regarding a potential role of the PALB2 c.1592delT mutation in the response of breast cancer patients to conventional chemotherapies, we observed increased levels of 53BP1 in healthy PALB2 c.1592delT mutation carriers but not in breast cancer patients. Together with RIF1 and PTIP, 53BP1 was reported to prevent unscheduled resection of DSBs, particularly in BRCA1-deficient cells. Consistent with a role of 53BP1 in NHEJ,51 53BP1 knockdown downregulated NHEJ in both the cohort of PALB2-mutated healthy individuals as well as in breast cancer patients. However, 53BP1 was limiting for MMEEJ in breast cancer patients in particular. Our results further suggested that RAD52 may contribute to SSA and MMEEJ in healthy mutation carriers, while RAD52-independent SSA and MMEEJ mechanisms53 may have a more important role in patients. Altogether, we found differential involvement of DSB repair components in compensatory error-prone DSB repair in healthy mutation carriers and breast cancer patients. Importantly, in triple-negative breast cancer, which is frequently associated with a nonfunctional BRCA1 and FA pathway, 53BP1 was found to be downregulated in a subset of the cases associated with reduced therapeutic responsiveness and antibiotic pathway usage was even more significant in the cohort of PALB2 mutation carriers (without therapy) regarding the replication stress as a source of DSBs in breast cancer patients with a PALB2 c.1592delT mutation.

Functional assays gradually enter the clinic to contribute to the classification of variants of uncertain significance in BRCA1 and BRCA2.36,57 Our previous work has taken functional analysis one step further demonstrating DSB repair dysregulation directly in immortalized and primary cells derived from individuals with hereditary breast cancer risk.36 In this study, we observed haploinsufficiency in PALB2 c.1592delT mutation carriers (without therapy) regarding the suppression of error-prone DSB repair, however, not regarding HR changes, RAD51 filament assembly or PARP inhibitor sensitivity. Thus, mutation carriers exhibited significantly increased MMEEJ and SSA activities. This shift in DSB repair pathway usage is particularly detrimental in the context of the aberrant replication phenotype observed previously in the same PALB2 c.1592delT mutation cohort cells.32 We consider the replication stress as a source of DSBs in combination with the increased error-prone DSB repair described here sufficient to explain the increase in genetic aberrations including complex chromosomal rearrangements in PALB2 c.1592delT mutation carrier cells.32 The difference in DSB repair pathway usage was even more significant in the PALB2 mutation carriers with breast cancer, underscoring the potential of the functional approach to capture additional risk factors. Intriguingly, in the breast cancer patients, we noticed the loss of 53BP1 accumulation, which has prognostic value in triple-negative breast cancer patients. Combining analysis of error-prone DSB repair activities and of 53BP1 levels may contribute to multifactorial models to predict the pathogenicity of individual risk genes and/or therapeutic responses.

**MATERIALS AND METHODS**

**LCLs and cell culturing**

LCLs originating from seven female Finnish PALB2 c.1592delT mutation carriers and six wild-type matched controls (Supplementary Table 1) were provided by the Laboratory of Cancer Genetics and Tumor Biology, University of Oulu, Finland, and described in the study by Nikkilä et al. Additional LCLs were HA238 with heterozygously mutated BRCA2 (provided by Medical University Hannover, Germany), GM13023A with biallelic BRCA2/FANCD1-mutation (purchased at Coriell Institute, Camden, NJ, USA), and the control LCL TK6 provided by Eppendorf University Clinic, Hamburg, Germany), which were all described in the study by Keimling et al. LCLs were cultivated in RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Biochrom, Merck Millipore, Darmstadt, Germany) and antibiotics (Penicillin-Streptomycin-Glutamine, Gibco/Invitrogen). Experiments were performed in antibiotic-free media with 1.5% L-glutamine (Gibco/Invitrogen). All LCLs were tested negative for Mycoplasma contamination.

**DSB repair analysis**

LCLs were analyzed in sets, whereby the reference LCL BR-096B was included in all sets. The capacity and quality of DSB repair was analyzed by use of a previously described EJ-based test system. For this purpose, LCLs were electroporated with a plasmid mixture containing 10 μg of one of the DSB repair substrates (EJScceGFP, EJ-EGFP, 5′EGFP/HR-EGFP, HR-EGFP/3′EGFP, HR-EGFP/5′EGFP) together with 10 μg of the meganuclease expression plasmid pCMV-1-I-sce, and 10 μg of the filler plasmid pBS by use of Gene Pulser (Bio-Rad, Person, Germany) at 2200 V and 1050 μF. To specify transfection efficiencies, wild-type EGFP expression plasmid was used instead of pBS in parallel samples each. Forty-eight hours post transfection, green fluorescent cells containing reconstituted EGFp were quantified by flow cytometry using FACS Calibur (Becton-Dickinson, Heidelberg, Germany). Laser excitation was effected at 488 nm and gated life cells (side scatter/forward scatter dot plot) were detected in the FL1/FL2 channel. The recombinase frequency was calculated by green fluorescent cells per life cell count divided by the transfection efficiency per life cell count. In experiments analyzing exogenous PALB2 protein, we added 40 μg of the POZ-PALB2 expression plasmid for HA-tagged wild-type PALB232 or of the expression plasmid for the tagged truncated variant encoded by PALB2 c.1592delT (Origene, Rockville, MD, USA). To silence 53BP1 during DSB repair measurements, we included 40 μg of two specific siR-based shRNA expression plasmids (Origene) into the mixture, to express RAD51SM pcDNA3.1-Rad51SM, to silence RAD52 pSUPER-RAD52.

Our study followed the guideline by Guidugli et al. of assay development for BRCA2 mutation carriers. Thus, we used an internal reference, replicated each sample, repeated each experiment at least twice, measured the transfection efficiency and prepared plasmids in batches to assure the same DNA quality. During assay optimization, we added further quality criteria to the present study, namely a minimum transfection efficiency for HR (≥10%), maximum LCL passage numbers (≤25) and performed the whole study in a blinded manner.

**Immunofluorescence microscopic analysis**

LCLs were exposed to 2 Gy of ionizing radiation (Cs-137, GSR D1, Gamma-Service Medical GmbH, Leipzig, Germany) or treated with MMC (2.6 μM; Sigma-Aldrich, Steinheim, Germany) for 45 min, reincubated with fresh medium and harvested at the indicated time points post treatment. After cytospinning (Cytospin3 Centrifuge, Shandon, Bohemia, NY, USA) at 28 × g for 5 min on slides covered by poly-L-Lysine (Sigma-Aldrich), cells were, depending on the specific antibody applied, pre-extracted or fixed immediately with 3.7% formaldehyde followed by permeabilization with 0.5% TritonX-100. Five percent Goat Serum (Invitrogen, Karlsruhe, Germany) in phosphate-buffered saline was utilized for blocking. Primary antibodies used were polyclonal antibodies targeting 53BP1 (NB100-304, Novus Biologicals, Littleton, CO, USA) and RAD51 (H-92, Santa Cruz Biotechnology, Heidelberg, Germany); secondary antibody was AlexaFluor555-labeled (Invitrogen). Stained cells were mounted either with Dabco (1,4-Diazabicyclo[2.2.2]-octane, Sigma-Aldrich) and Mowiol (MOWIOL 48–88 Reagent, Calbiochem, Merck Millipore) after staining with DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich) or Vectashield containing DAPI (Vector laboratories, Burlingame, CA, USA). Focal accumulation of 53BP1 and RAD51 in the nuclei was analyzed in a time-dependent manner using an Olympus BX51 epifluorescence microscope with ×100 oil immersion objective fitted with cooled charge-coupled device camera (Colorview 12, Olympus, Tokyo, Japan) and Cell–F imaging software version 2.5 (Olympus Soft Imaging Solutions, Münster, Germany). On each experimental day, 50 nuclei from two independent slides were analyzed. The threshold was maintained throughout one experimental set.

**PARP inhibitor sensitivity**

To assess the response to the PARP inhibitor 1,5-isoquinolinediol (ENZO, New York, NY, USA), MTT assay was performed as described.59 One day post seeding, LCLs were treated with different 1,5-isoquinolinediol concentrations from 2 μM to 2 mM 1,5-isoquinolinediol for 6 days.
Western blotting

LCLs were mock-treated or treated with MMC (2.6 μM) for 45 min followed by incubation in fresh media and harvested at the indicated time points. After preparation of the lysates, proteins were electrophoresed by SDS-PAGE and transferred to nitrocellulose or polyvinylidene fluoride membrane. Proteins were detected by the following antibodies recognizing 33BP1 (NB100-304, Novus Biologicals, Cambridge, UK), CHIP (T-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA), MRE11 (NB100-142, M-2; Novus Biologicals), RAD51 (H-92, Santa Cruz Biotechnology), RAD52 (SH9, Abcam, Cambridge, UK), RPA (Ab-2, Calbiochem, Darmstadt, Germany), phospho-HPA (P-RPA; S33, Bethyl Laboratories, Montgomery, TX, USA), PARP1 (Cambridge, UK), RPA (Ab-2, Calbiochem, Darmstadt, Germany), phospho-Cruz Biotechnology, Santa Cruz, CA, USA), MRE11 (NB100-142, M-2; Novus Biologicals), 53BP1 (NB100-304, Novus Biologicals, Cambridge, UK), CtIP (T-16, Santa Cruz, CA, USA) and α-Tubulin (ab7291, Abcam). Secondary anti-bodies were peroxidase-conjugated goat anti-mouse (Thermo Scientific, Waltham, MA, USA), anti-rabbit and anti-goat IgG (Rockland, Gilbertsville, PA, USA). Bands were visualized with Clarity Western ECL Substrate (Bio-Rad Laboratories) by ChemiDoc MP Imaging System and quantified using Image Lab 4.1 (Bio-Rad Laboratories).

Statistical evaluation

The statistical significances of differences for set-wise DS8 repair measurements, foci analysis and Spearman correlation were determined by the software GraphPad Prism 5.04 (GraphPad, San Diego, CA, USA). Once statistical significance was established for a data set by Kruskal–Wallis test, non-parametric Mann–Whitney test for unpaired samples was applied. GraphPad Prism 5.04 software was further used to generate cell viability curves, calculate IC50 values and statistical significances of the differences between the IC50 values by Extra sum-of-squares F-test of Log IC50. Cohort analyses of DS8 repair pathway usage and DNA damage signals were performed using IBM SPSS Statistics Software package version 21. DS8 repair pathway usage was analyzed using a general linear mixed model with mutation/health status as fixed factor and date of experiment as random factor. DNA damage signals were analyzed using general linear models with mutation/health status and treatment group (untreated control, 0 or 1 hr post treatment, 6 hr post treatment) as fixed factors. Post hoc tests were adjusted for multiple comparisons using Bonferroni correction. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

CONFIDENT OF INTEREST

The authors declare no conflict of interest. LW is an inventor of a patent on a test system for determining genotoxicity, which is owned by LW.

ACKNOWLEDGEMENTS

We cordially thank Leena Keskitalo, Annika Väntänen and Meeri Otsukka for the initial phase of the project, and Jeremy M Stark, Department of Cancer Biology, the current study. We are grateful to Daniela Salles, Ulm, for experimental help during the initial phase of the project, and Jeremy M Stark, Department of Cancer Biology, the current study. We are grateful to Daniela Salles, Ulm, for experimental help during the initial phase of the project, and Jeremy M Stark, Department of Cancer Biology, the current study. We are grateful to Daniela Salles, Ulm, for experimental help during the initial phase of the project, and Jeremy M Stark, Department of Cancer Biology, the current study. We are grateful to Daniela Salles, Ulm, for experimental help during the initial phase of the project, and Jeremy M Stark, Department of Cancer Biology, the current study.
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