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Original research

Characterisation of protein-truncating and missense variants in *PALB2* in 15 768 women from Malaysia and Singapore

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ABSTRACT

Background Rare protein-truncating variants (PTVs) in partner and localiser of BRCA2 (*PALB2*) confer increased risk to breast cancer, but relatively few studies have reported the prevalence in South-East Asian populations. Here, we describe the prevalence of rare variants in *PALB2* in a population-based study of 7840 breast cancer cases and 7928 healthy Chinese, Malay and Indian women from Malaysia and Singapore, and describe the functional impact of germline missense variants identified in this population.

Methods Mutation testing was performed on germline DNA (n=15 768) using targeted sequencing panels. The functional impact of missense variants was tested in mouse embryonic stem cell based functional assays.

Results PTVs in *PALB2* were found in 0.73% of breast cancer patients and 0.14% of healthy individuals (OR=5.44; 95% CI 2.85 to 10.39, p<0.0001). In contrast, rare missense variants in *PALB2* were not associated with increased risk of breast cancer. Whereas PTVs were associated with later stage of presentation and higher-grade tumours, no significant association was observed with missense variants in *PALB2*. However, two novel rare missense variants (p.L1027R and p.G1043V) produced unstable proteins and resulted in a decrease in homologous recombination-mediated repair of DNA double-strand breaks.

Conclusion Despite genetic and lifestyle differences between Asian and other populations, the population prevalence of *PALB2* PTVs and associated relative risk of breast cancer, are similar to those reported in European populations.

INTRODUCTION

PALB2 (partner and localiser of BRCA2) plays a vital role in maintenance of genome integrity and repair of DNA double-strand breaks via a homologous recombination (HR) pathway, by localising BRCA2 to the sites of DNA damage and serving as a linker between BRCA1 and BRCA2.[1 2] Bi-allelic (homozygous) germline truncating mutations in *PALB2* result in Fanconi anaemia,[3] whereas mono-allelic (heterozygous) truncating mutations predispose individuals to breast, ovarian and pancreatic cancers.[4 5]

Protein-truncating variants (PTVs) in *PALB2* have been shown to be associated with fivefold to sevenfold increase in risk to breast cancer in women of European and Asian descent.[5–8] However, less is known about missense variants, especially variants found in understudied populations. Notably, unlike *BRCA1* and *BRCA2* where there have been extensive efforts to characterise the functional impact of missense variants, including using saturation genome editing approaches, multiplex homology directed repair assays and validated transcriptional assays,[9–12] there have been fewer reports on the functional characterisation of missense variants in *PALB2*.[13–17]

In this study, we report the prevalence of rare variants in *PALB2* in 7840 patients with breast cancer and 7928 healthy controls from Malaysia and Singapore, and contrast the clinicopathological features of *PALB2* variant carriers with those of *BRCA1* and *BRCA2* carriers, and non-carriers. We report the functional characterisation of rare missense variants by performing functional analyses in mouse embryonic stem (mES) cells.

METHODS

Study subjects

The study participants were women recruited in the Malaysian Breast Cancer Genetic Study (MyBrCa) [18] and the Singapore Breast Cancer Cohort Study (SGBCC). Cases were recruited from two hospitals in Malaysia (recruitment started in 2002 in the first hospital and extended to the second hospital in 2012) and six hospitals in Singapore (recruitment started in 2010 in the first hospital and extended to additional five hospitals by 2016). Prevalent and incident breast cancer cases, both invasive and non-invasive, were included.

In MyBrCa, controls were healthy women between ages 40 years and 74 years, with no personal history of breast cancer, recruited through a subsidised opportunistic mammography screening programme that was initiated in the same two hospitals where cases were recruited. The Singaporean controls were unaffected individuals from the Singapore Population Health Studies (National University Health System, 2016) and the Singapore Multi-Ethnic Cohort,[19] and individually matched by ethnicity and age ±5 years to the SGBCC cases.

Clinical data were retrieved from hospital records: Her2 scores of 0 and 1+ were considered 'negative', those with 2+ by immunohistochemistry (IHC) and amplification by fluorescence in situ hybridisation/silver in situ hybridisation or 3+ by IHC alone were considered 'positive'. In MyBrCa, family history of all cancers was collected and in SGBCC, only information on first degree family history of breast or ovarian cancer was collected.

Participants donated a blood or saliva sample that was processed and stored, completed a questionnaire that included information on lifestyle risk factors for breast cancer, and provided written informed consent.

Sequencing and bioinformatics analysis

Germline DNA of cases and controls were sequenced in two batches, using targeted sequencing panels that target the coding regions and exon-intron boundaries of known and suspected breast cancer susceptibility genes, respectively, which included *PALB2*, *BRCA1* and *BRCA2* genes.[7 8 20] Target enrichment were performed using the Fluidgm Access Array system (n=5090) or the Fluidgm Juno system (n=11 342) and sequenced on Illumina HiSeq 2500 or HiSeq 4000. Specifically, the 11 342 samples analysed on the Fluidgm Juno system were described in Dorling *et al*.[8] As *PALB2* is a relatively rare breast cancer gene, we have combined both analyses in this paper and further characterised the role of missense variants in this population, which has previously not been reported. Library preparations were performed according to manufacturer's protocols as described previously.[7 8 20] In total, germline DNA from 8205 breast cancer patients and 8227 controls were analysed by panel sequencing. After excluding samples that failed sequencing quality control, 7840 cases and 7928 controls were included for subsequent analyses (online supplemental table 1).

Analysis of sequencing data was performed as described previously.[8 20] Briefly, raw sequence data were demultiplexed and aligned to the human reference genome, hg19 using BWA-MEM.[21 22] Variant calling was performed using VarDict.[23] Analyses were restricted to putative PTVs and rare missense variants. All frameshift, stop-gain (nonsense) and consensus splice site variants were considered as PTVs unless reported otherwise by the Evidence-based Network for the Interpretation of Germline Mutant Alleles consortium.[24 25] Rare missense variants were defined as having a minor allelic frequency <0.1% present in gnomAD. All PTVs and rare missense variants annotated by the align-GVGD (http://agvgd.iarc.fr) in silico tool as likely pathogenic (C15–C65) were validated by Sanger sequencing. NM_024675.3 was used as the reference sequence for *PALB2* variants.

Functional analysis of rare germline *PALB2* missense variants

Functional analysis on *PALB2* missense variants was performed using several methods as previously described.[15] First, the HR reporter assay was performed in *Trp53*^{KO}/*Palb2*^{KO} mES cells which were complemented with human *PALB2* variants (or an empty vector, Ev). Two days after transfection of an *I-SceI* and mCherry coexpression vector,[26] GFP expression was measured using fluorescence-activated cell sorting (FACS). A proliferation-based PARP inhibitor (PARPi; Selleckchem S1060) sensitivity assay was performed using *Trp53*^{KO}/*Palb2*^{KO} mES cells for five *PALB2* missense variants that exhibited the largest defect in DR-GFP assays. Cells were exposed to various concentrations of PARPi for 2 days. Thereafter, cells were incubated for one more day in drug-free media, after which viability was measured using FACS (using only forward scatter and side scatter). Expression of all *PALB2* variants was examined by western blot analysis. Two different primary rabbit polyclonal antibodies directed against the N-terminus of human PALB2 (1:1000, kindly provided by Cell Signalling Technology prior to commercialisation) were used. Wild type human *PALB2* and Ev were used as controls on the blot while tubulin (Sigma, T6199 clone DM1A) was used as loading control. Lastly, RT-qPCR was performed for a selected panel of *PALB2* variants. Briefly, RNA was isolated using Trizol (ThermoFisher, 15596026) and DNAse (Promega, M6101). Subsequently, reverse transcriptase (ThermoFisher, 12328019) reactions were performed as previously described.[15] GoTaq qPCR Master mix (Promega, A6002) and the following qPCR primers directed at the human PALB2 cDNA or the mouse control gene Pim1 were used; human PALB2-Fw— 5'-GATTACAAGGAT GACGACGATAAGATGGAC-3', human PALB2-Rv—5'-CCTT TTCAAGAATGCTAATTTCTCCTTTAACTTTTCC-3', mouse Pim1-exon4-Fw—5'-GCGGCGAAATCAAACTCATCGAC-3' and mouse Pim1-exon5-Rv—5'-GTAGCGATGGTAGCGA ATCCACTCTGG-3'.

For protein stability and degradation assays, cells were treated with 100 µg/mL cycloheximide (Sigma, C7698-1G) for up to 3 hours, or 0.5 or 3 µM MG-132 (Selleckchem, S2619) for 24 hours, after which western blot samples were collected and analysed. Quantification of EGFP-PALB2 subcellular localisation was based on transient expression in HeLa cells that were fixed using 4% formaldehyde and permeabilised using Triton X-100. Cells were immunostained with anti-GFP and DAPI prior to immunofluorescence analysis and quantification (based on ~25 cells per condition per replicate). All the aforementioned experiments were conducted in duplicate and average values and SEM were calculated to generate the respective plots.

Statistical analysis

Multivariable logistic regression was used to determine the association of pathogenic and missense variants with breast cancer risk, adjusting for age, batch of germline panel sequencing and country. Rare missense variants were further subcategorised based on domain and functional prediction scores using five in silico tools (align-GVGD, REVEL, VEST4, ClinPred and CADD). The clinicopathological characteristics of mutation carriers and non-carriers were compared using χ^2 test or Fisher's exact test, where appropriate, for categorical variables and t-test for continuous variables. Statistical analyses were performed using R V.3.6.1.

RESULTS

Germline PTVs and rare missense variants

A total of 57 (0.73%) cases and 11 (0.14%) healthy controls carried a pathogenic, protein-truncating, *PALB2* variant (OR=5.44, p<0.001; figure 1, table 1A). The estimated OR was, however, lower than for *BRCA1* (OR=10.68, p<0.001) or *BRCA2* (OR=15.61, p<0.001) PTVs. PTVs were distributed along the entire coding region of the gene (table 1A). Of the 34

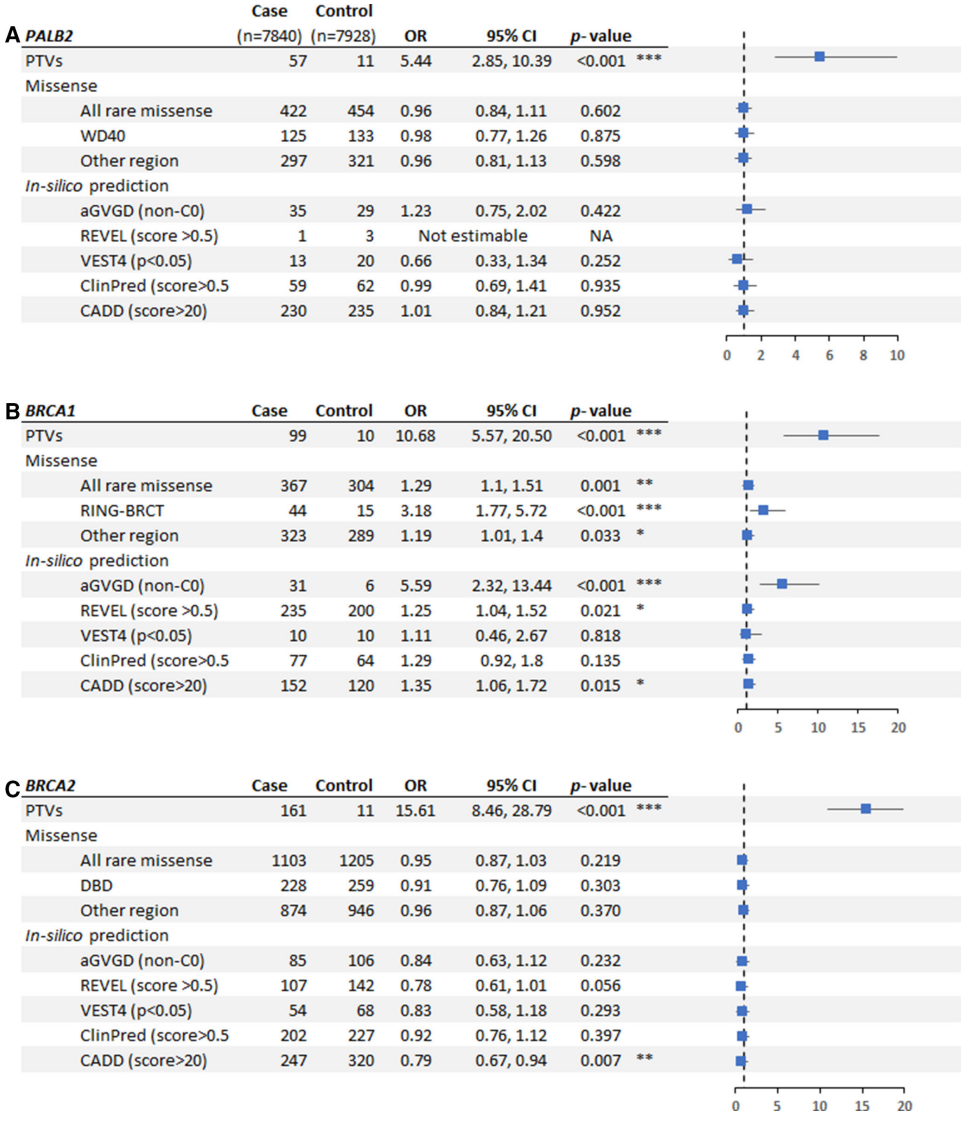

Figure 1 Association of protein-truncating variants (PTVs) and rare missense variants in *PALB2* (A), *BRCA1* (B) and *BRCA2* (C) with breast cancer risk. Missense variants were evaluated as a group for those located in functional domains and for those predicted to be likely pathogenic by in silico algorithms. WD40 (WD40 repeat domain), RING-BRCT (RING finger domain and BRCA1 C terminus), DBD (DNA binding domain), Align-GVGD (AGVGD), variants with score >C15, REVEL (score >0.5), VEST4 (p<0.05), ClinPred (score >0.5), CADD (score >20). PALB2, partner and localiser of BRCA2.

unique *PALB2* PTVs identified, five were identified in at least four individuals in our study: p.E3X, c. 211+1G>A, p.K346fs, p.V870X and p.E990X. These represented 44% of all *PALB2* PTV carriers. Notably, 24% (8/34) of the variants have not been reported in any of the public databases including ClinVar, gnomAD and LOVD (table 1A).

We identified 422 carriers of *PALB2* rare missense variants in cases and 454 carriers in healthy women (OR=0.96, p=0.602) (figure 1). No associations were observed when analysis was restricted to variants with higher scores using any of the five in silico tools tested (figure 1). There was also no evidence of an association with risk for variants specifically in the WD40 domain. These results contrast with those for *BRCA1*, where there is an overall association with breast cancer risk for rare missense variants (OR=1.29, p=0.001), an effect that is driven by rare missense variants in the RING and BRCT domains (OR=3.18, p<0.001). In addition, for *BRCA1* the risk was higher for variants with Align-GVGD C15–C65 scores (OR=5.59, p<0.001; figure 1). In *PALB2*, the frequency of Align-GVGD C15–C65 was slightly, but not significantly higher in cases than controls (35 carriers in cases and 29 carriers in controls). The 18 unique missense variants in this category were all located in functional domains or motifs. Five variants were recurrent and present in at least four individuals: p.G401R, p.P405A, p.S896F, p.T993M and p.T1012I represented 70% of all *PALB2* rare missense variant carriers (with AGVGD scores of C15 and above) in this cohort. Notably, 39% (7/18) of the variants were novel and have not been reported previously in public databases (table 1B).

Characteristics of germline carriers of *PALB2*, *BRCA1* and *BRCA2* PTVs and missense variants

In our study, 57 (0.73%), 99 (1.26%) and 161 (2.05%) patients with breast cancer had germline PTVs in *PALB2*, *BRCA1* and *BRCA2*, respectively (table 2); none had pathogenic variants in more than one gene. The distribution of age at diagnosis in *PALB2* was similar to that in non-carriers (mean age at diagnosis 51.3 years vs 52.5 years). This contrasts with *BRCA1* and *BRCA2*, where the carrier cases occurred at a young age (mean 44.1 years and 47.3 years, respectively). A family history of breast cancer

Table 1 List of *PALB2* variants identified

A.Protein-truncating variants (PTVs)

No	Type of mutation	cDNA change	AA change	Domain	Cases	Controls	Total	Previously reported
1	Nons	c.7G>T	p.E3X		5	0	5	Yes
2	SS	c.48+2T>G	–		0	1	1	Yes
3	Nons	c.73A>T	p.K25X	CC	1	0	1	Yes
4	SS	c.109–1G>A	–		1	0	1	No
5	SS	c.109-2A>G	-		1	0	1	Yes
6	SS	c.211+1G>A	–		4	3	7	Yes
7	FS delins	c.336_337delinsA	p.P113fs		3	0	3	No
8	FS del	c.426_428delinsCC	p.K142fs		1	0	1	No
9	Nons	c.751C>T	p.Q251X		0	1	1	Yes
10	Fs del	c.839del	p.N280fs		1	0	1	Yes
11	FS ins	c.886dup	p.M296fs		0	1	1	Yes
12	Fs del	c.1037_1041del	p.K346fs		4	0	4	Yes
13	Nons	c.1042C>T	p.Q348X		1	0	1	Yes
14	Fs del	c.1050_1053del	p.T351fs		2	0	2	Yes
15	Fs del	c.1056_1057del	p.K353fs		1	0	1	Yes
16	FS del	c.1059del	p.K353fs		3	0	3	Yes
17	FS del	c.1133del	p.P378fs		1	0	1	No
18	FS ins	c.1158dup	p.S387fs		1	0	1	No
19	Nons	c.1543A>T	p.K515X		1	0	1	No
20	FS del	c.1592del	p.L531X		0	1	1	Yes
21	FS del	c.1783del	p.D595fs	MBD	1	0	1	Yes
22	Fs del	c.1976_1977del	p.L659fs		1	0	1	No
23	Nons	c.2012T>G	p.L671X		1	0	1	Yes
24	Fs del	c.2167_2168del	p.M723fs		3	0	3	Yes
25	Nons	c.2257C>T	p.R753X		1	0	1	Yes
26	Nons	c.2336C>G	p.S779X		1	0	1	Yes
27	FS del	c.2607del	p.V870X	WD40	3	1	4	Yes
28	FS ins	c.2760dup	p.Q921fs	WD40	1	0	1	Yes
29	Nons	c.2968G>T	p.E990X	WD40	8	2	10	Yes
30	SS	c.3114–1G>A	–		1	1	2	Yes
31	FS del	c.3143del	p.K1048fs	WD40	1	0	1	Yes
32	Nons	c.3166C>T	p.Q1056X	WD40	1	0	1	Yes
33	Nons	c.3256C>T	p.R1086X	WD40	1	0	1	Yes
34	FS del	c.3543del	p.F1181fs	WD40	2	0	2	No
			Total		**57**	**11**	**68**	

B.Rare missense variants*

No	AGVGD score	cDNA change	AA change	Domain	Cases	Ctrls	Total	Previously reported
1	C25	c.25C>G	p.L9V	CC	1	0	1	No
2	C65	c.109C>T	p.R37C	CC	1	2	3	Yes
3	C25	c.110G>A	p.R37H	CC	1	0	1	Yes
4	C15	c.116A>T	p.Q39L	CC	1	0	1	Yes
5	C65	c.1201G>C	p.G401R	ChAM	1	3	4	No
6	C25	c.1213C>G	p.P405A	ChAM	5	5	10	Yes
7	C65	c.1226A>G	p.Y409C	ChAM	1	1	2	Yes
8	C15	c.1255T>C	p.C419R	ChAM	2	1	3	No
9	C65	c.1843C>T	p.P615S	MBD	0	1	1	Yes
10	C15	c.2687C>T	p.S896F	WD40	4	0	4	No
11	C15	c.2978C>T	p.T993M	WD40	4	1	5	Yes
12	C15	c.3035C>T	p.T1012I	WD40	9	13	22	Yes
13	C35	c.3080T>G	p.L1027R	WD40	1	0	1	No
14	C25	c.3107T>C	p.V1036A	WD40	2	0	2	Yes
15	C65	c.3128G>T	p.G1043V	WD40	1	0	1	No
16	C15	c.3132A>T	p.Q1044H	WD40	0	1	1	Yes
17	C15	c.3506C>G	p.S1169C	WD40	0	1	1	Yes
18	C15	c.3549_3552delinsTTTG	p.H1184L	WD40	1	0	1	No
			Total		**35**	**29**	**64**	

Reference sequence: NM_024675.3.
*, variants with AGVGD scores of C15 and above; CC, coiled-coil; PALB2, partner and localiser of BRCA2.

Table 2 Clinical and demographic characteristics of carriers with protein-truncating variants

Variable	*PALB2* carriers (n=57)	*BRCA1* carriers (n=99)	*BRCA2* carriers (n=161)	Non-carriers (n=7523)	P value*	P value†	P value‡
Age at diagnosis (mean±SD)	51.3±10.7	44.1±10.8	47.3±10.5	52.5±10.7	0.414	<0.001	<0.001
Age distribution (years)					0.612	<0.001	<0.001
<30	2 (3.5)	7 (7.1)	4 (2.5)	101 (1.4)			
30–39	6 (10.5)	30 (30.0)	35 (21.9)	672 (9.0)			
40–49	16 (28.1)	34 (34.7)	59 (36.9)	2260 (30.2)			
50–59	18 (31.6)	17 (17.3)	40 (25.0)	2538 (33.9)			
>60	15 (26.3)	10 (10.2)	22 (13.8)	1907 (25.5)			
Ethnicity					0.728	0.003	0.021
Chinese	41 (73.2)	59 (59.6)	104 (64.6)	5696 (75.8)			
Malay	11 (19.6)	25 (25.3)	36 (22.4)	1088 (14.5)			
Indian	4 (7.1)	14 (14.1)	20 (12.4)	651 (8.7)			
Other	0 (0.0)	1 (1.0)	1 (0.6)	79 (1.1)			
Family history of breast cancer, first deg					0.087	<0.001	<0.001
Yes	13 (22.8)	38 (38.8)	47 (29.4)	1071 (14.4)			
No	44 (77.2)	60 (61.2)	113 (70.6)	6344 (85.6)			
Family history of ovarian cancer, first deg					0.551	<0.001	0.029
Yes	1 (2.1)	13 (14.9)	7 (4.8)	108 (1.6)			
No	47 (97.9)	74 (85.1)	138 (95.2)	6463 (98.4)			
Bilaterality					0.500	0.001	0.008
Yes	3 (5.4)	12 (12.2)	14 (8.8)	306 (4.1)			
No	53 (94.6)	86 (87.8)	145 (91.2)	7169 (95.9)			
Tumour stage					0.002	0.228	0.005
Stage 0	0 (0.0)	5 (6.7)	6 (4.7)	698 (11.2)			
Stage I	6 (15.0)	19 (25.3)	30 (23.6)	1965 (31.6)			
Stage II	22 (55.0)	30 (40.0)	54 (42.5)	2338 (37.6)			
Stage III	11 (27.5)	18 (24.0)	27 (21.3)	966 (15.5)			
Stage IV	1 (2.5)	3 (4.0)	10 (7.9)	248 (4.0)			
Tumour grade					0.045	<0.001	<0.001
Low	2 (4.2)	2 (2.6)	3 (2.2)	950 (14.8)			
Intermediate	20 (41.7)	19 (24.4)	65 (47.8)	2847 (44.3)			
High	26 (54.2)	57 (73.1)	68 (50.0)	2623 (40.9)			
ER status					0.278	<0.001	0.412
Positive	34 (65.4)	21 (24.1)	104 (72.7)	4833 (72.3)			
Negative	18 (34.6)	66 (75.9)	39 (27.3)	1854 (27.7)			
PR status					0.055	<0.001	0.328
Positive	25 (50.0)	19 (22.6)	84 (60.9)	4117 (63.7)			
Negative	25 (50.0)	65 (77.4)	54 (39.1)	2350 (36.3)			
HER2 status					0.630	0.001	<0.001
Positive	12 (26.1)	11 (13.9)	19 (16.2)	1695 (30.7)			
Negative	34 (73.9)	68 (86.1)	98 (83.8)	3820 (69.3)			
Triple negative breast cancer					0.266	<0.001	0.029
Yes	8 (17.8)	49 (64.5)	24 (20.9)	677 (12.6)			
No	37 (82.2)	27 (35.5)	91 (79.1)	4688 (87.4)			
Study					0.006	0.014	0.023
MyBrCa	35 (61.4)	55 (55.6)	84 (52.2)	3249 (43.2)			
SGBCC	22 (38.6)	44 (44.4)	77 (47.8)	4274 (56.8)			

**PALB2* mutation carriers versus non-carriers.
†*BRCA1* mutation carriers versus non-carriers.
‡*BRCA2* mutation carriers versus non-carriers.
MyBrCa, Malaysian Breast Cancer Genetic Study; PALB2, partner and localiser of BRCA2; SGBCC, Singapore Breast Cancer Cohort Study.

was more common in *PALB2* carriers than in non-carriers, but not significantly so. There was no association with personal or family history of pancreatic cancer, or family history of male breast cancer, where information was available (data not shown).

Notably, there was no significant difference in the crude prevalence of *PALB2* carriers among Chinese, Malay and Indian patients (0.7%, 1.0% and 0.6%, respectively), but there was a higher prevalence of *BRCA1* and *BRCA2* variants in Malay and Indian patients compared with Chinese patients (2.2% and 2.0% compared with 1.0% for *BRCA1*, and 3.1% and 2.9% compared with 1.8% for *BRCA2*). There was no significant association with ER or HER2 status, but an association with PR-negative disease was of borderline significance (table 2, figure 2). We observed a higher prevalence of *PALB2* carriers in the Malaysian cohort, but this was not statistically significant after adjustment for stage and grade in the multivariable analysis. Similarly, there was a

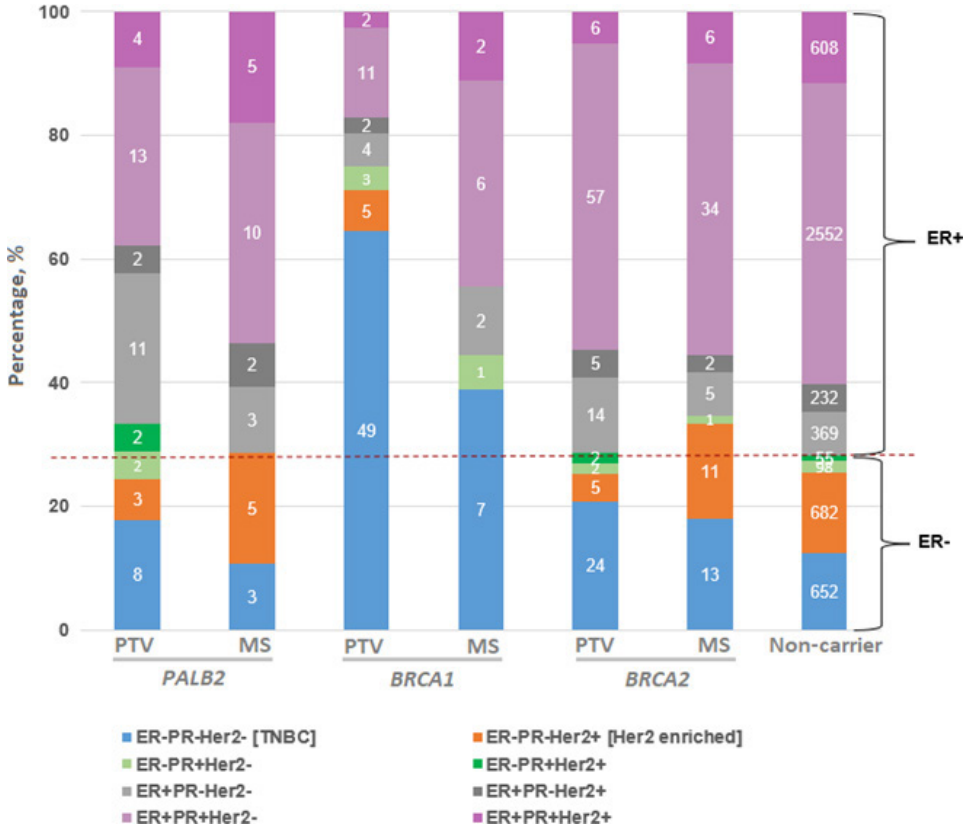

Figure 2 Distribution of breast cancer subtypes by immunohistochemistry (IHC): the stacked bar chart compares the distribution of tumour subtypes with germline alterations (protein-truncating variant (PTV) or missense (MS) variants with AGVGD scores of C15 and above) in *PALB2* with *BRCA1*, *BRCA2* and tumours with no alterations that arise from non-carriers. The horizontal dotted line indicates the proportion of ER negative breast cancer among the non-carriers. PALB2, partner and localiser of BRCA2.

higher prevalence of *BRCA1* and *BRCA2* carriers in the Malaysian cohort, but this was not statistically significant after adjustment for age and ethnicity in the multivariable analysis.

There were 35 (0.45%), 31 (0.40%) and 85 (1.08%) patients with breast cancer with a likely pathogenic missense variant in *PALB2*, *BRCA1* and *BRCA2*, respectively, as predicted by the Align-GVGD algorithm. Like PTV carriers, *BRCA1* rare missense carriers were more likely to develop breast cancer at a significantly younger age when compared with the non-carriers (47.5 years old vs 52.5 years old). However, there was no significant difference in age of diagnosis in carriers of *PALB2* rare missense variants compared with non-carriers (table 3).

We examined the distribution of breast cancer subtypes of carriers of rare missense variants by IHC assessment and found that, similar to carriers of pathogenic variants in *BRCA1*, carriers of rare missense variants in *BRCA1* appear to be more likely to develop high grade tumours and triple negative subtype (table 3, figure 2). By contrast, there was no significant difference in the distribution of breast cancer subtypes in carriers of rare missense variants in *PALB2* compared with non-carriers (figure 2).

Functional characterisation of *PALB2* rare missense variants

As computational approaches for predicting the effects of missense variants often produce conflicting results,[10 15 16] we evaluated the functional impact of the missense variants in our previously published mES cell-based functional assay.[15] Briefly, mES cells in which *Palb2* has been deleted using CRISPR-Cas9 technology were complemented with human *PALB2* cDNA, with or without *PALB2* variant, through stable integration at the Rosa26 locus.[15] By using the well-established DR-GFP reporter,[27] which was integrated at the Pim1 locus, HR was measured to evaluate the functional impact of variants in PALB2.[15] In this study we evaluated 18 missense variants (with AGVGD score of ≥C15) as listed in table 1B and two other variants (p.A38G and p.A38V) with AGVGD score of C0 were included for comparison purposes. Of the 20 missense variants tested, 2 variants (p.R37C and p.R37H) exhibited moderate HR activity (50%–60%). Our data on p.R37C contrast those of a previous study,[16] showing that that this variant is fully functional. Complementation by transient overexpression of *PALB2* cDNA carrying this variant, versus complementation by stable integration, may explain this difference as discussed previously.[28] Our data are generally in agreement with previous studies showing that p.R37H exhibits a moderate impact on HR, although HR rates are slightly variable between the different studies.[14–17] An impaired PALB2-BRCA1 interaction likely explains this defect, as well as the reduced recruitment of p.R37H to sites of DNA damage induced by laser micro-irradiation.[15]

Interestingly, two other *PALB2* missense variants (p.L1027R and p.G1043V) exhibited a >80% reduction in HR (figure 3A), indicating that they are similarly damaging as truncating *PALB2* variants.[15] As HR defects have been associated with sensitivity to PARPis,[29] we evaluated the effect of five *PALB2* missense variants that exhibited the largest defect in HR in DR-GFP assays, using a cellular proliferation assay. We found that p.R37H and p.A38V did not have a major impact on PARP sensitivity, whereas p.L1027R and p.G1043V displayed strong sensitivity to PARP inhibition (figure 3B). Consistently, western blot analysis for all

Table 3 Clinical and demographic characteristics of carriers with rare missense variants

Variable	*PALB2* carriers (n=35)	*BRCA1* carriers (n=31)	*BRCA2* carriers (n=85)	Non-carriers* (n=7372)	P value†	P value‡	P value§
Age at diagnosis (mean±SD)	51.9±10.6	47.5±10.8	51.7±11.7	52.5±10.7	0.748	0.009	0.460
Age distribution (years)					0.705	0.086	0.273
<30	0 (0.0)	0 (0.0)	2 (2.4)	99 (1.4)			
30–39	5 (14.7)	7 (23.3)	9 (10.6)	651 (8.9)			
40–49	9 (26.5)	11 (36.7)	29 (34.1)	2211 (30.2)			
50–59	10 (29.4)	9 (30.0)	20 (23.5)	2499 (34.1)			
>60	10 (29.4)	3 (10.0)	25 (29.4)	1869 (25.5)			
Ethnicity					0.807	0.002	0.003
Chinese	27 (77.1)	17 (54.8)	55 (64.7)	5597 (76)			
Malay	4 (11.4)	13 (41.9)	11 (12.9)	1060 (14.4)			
Indian	4 (11.4)	1 (3.2)	17 (20.0)	629 (8.5)			
Other	0 (0.0)	0 (0.0)	2 (2.4)	77 (1.0)			
Family history of breast cancer, first deg					0.467	0.797	0.351
Yes	3 (8.8)	5 (16.1)	15 (17.9)	1048 (14.4)			
No	31 (91.2)	26 (83.9)	69 (82.1)	6218 (85.6)			
Family history of ovarian cancer, first deg					1.000	0.079	0.638
Yes	0 (0.0)	2 (7.1)	0 (0.0)	106 (1.6)			
No	28 (100.0)	26 (92.9)	77 (100.0)	6332 (98.4)			
Bilaterality					1.000	1.000	1.000
Yes	1 (2.9)	1 (3.2)	3 (3.6)	301 (4.1)			
No	34 (97.1)	30 (96.8)	80 (96.4)	7025 (95.9)			
Tumour stage					0.684	0.450	0.569
Stage 0	2 (7.1)	0 (0)	4 (5.7)	692 (11.4)			
Stage I	11 (39.3)	8 (36.4)	23 (32.9)	1923 (31.6)			
Stage II	12 (42.9)	10 (45.5)	28 (40.0)	2288 (37.5)			
Stage III	2 (7.1)	3 (13.6)	11 (15.7)	950 (15.6)			
Stage IV	1 (3.6)	1 (4.5)	4 (5.7)	242 (4.0)			
Tumour grade					0.855	0.010	0.252
Low	5 (16.1)	3 (11.5)	5 (7.8)	937 (14.9)			
Intermediate	15 (48.4)	5 (19.2)	29 (45.3)	2798 (44.4)			
High	11 (35.5)	18 (69.2)	30 (46.9)	2564 (40.7)			
ER status					1.000	0.168	1.000
Positive	23 (74.2)	14 (58.3)	57 (72.2)	4739 (72.3)			
Negative	8 (25.8)	10 (41.7)	22 (27.8)	1814 (27.7)			
PR status					0.575	0.829	0.546
Positive	18 (58.1)	14 (60.9)	45 (60.0)	4040 (63.7)			
Negative	13 (41.9)	9 (39.1)	30 (40.0)	2298 (36.3)			
HER2 status					0.229	0.610	0.424
Positive	12 (41.4)	4 (22.2)	17 (25.4)	1662 (30.8)			
Negative	17 (58.6)	14 (77.8)	50 (74.6)	3739 (69.2)			
Triple negative breast cancer					1.000	0.017	0.053
Yes	3 (8.6)	7 (22.6)	13 (15.3)	654 (8.9)			
No	32 (91.4)	24 (77.4)	72 (84.7)	6716 (91.1)			
Study					0.712	0.093	0.463
MyBrCa	14 (40.0)	18 (58.1)	40 (47.1)	3177 (43.1)			
SGBCC	21 (60.0)	13 (41.9)	45 (52.9)	4195 (56.9)			

*Non-carriers: Do not carry either protein-truncating or rare missense variants (with AGVGD scores of C15 and above) in three genes.
†*PALB2* mutation carriers versus non-carriers.
‡*BRCA1* mutation carriers versus non-carriers.
§*BRCA2* mutation carriers versus non-carriers.
MyBrCa, Malaysian Breast Cancer Genetic Study; PALB2, partner and localiser of BRCA2; SGBCC, Singapore Breast Cancer Cohort Study.

20 missense variants showed weak expression for p.L1027R and p.G1043V in comparison to that of wild type *PALB2* (figure 3C), suggesting that these two variants negatively affect PALB2 protein levels. mRNA analysis subsequently showed that the transcript levels of several variants, including p.L1027R and p.G1043V, were similar to that of the wild type complemented condition, suggesting that the weak expression of p.L1027R and p.G1043V is likely due to protein instability (figure 3D). To examine this further, we performed cycloheximide chase experiments to halt protein synthesis and assess PALB2 protein levels over time. While wild type PALB2 protein levels remained stable over a 3-hour time span after cycloheximide treatment, both p.L1027R and p.G1043V showed marked reductions in protein levels compared with the 0-hour time point (figure 3E). These data provide evidence that p.L1027R and p.G1043V impair PALB2 protein function through protein instability. Treatment with the

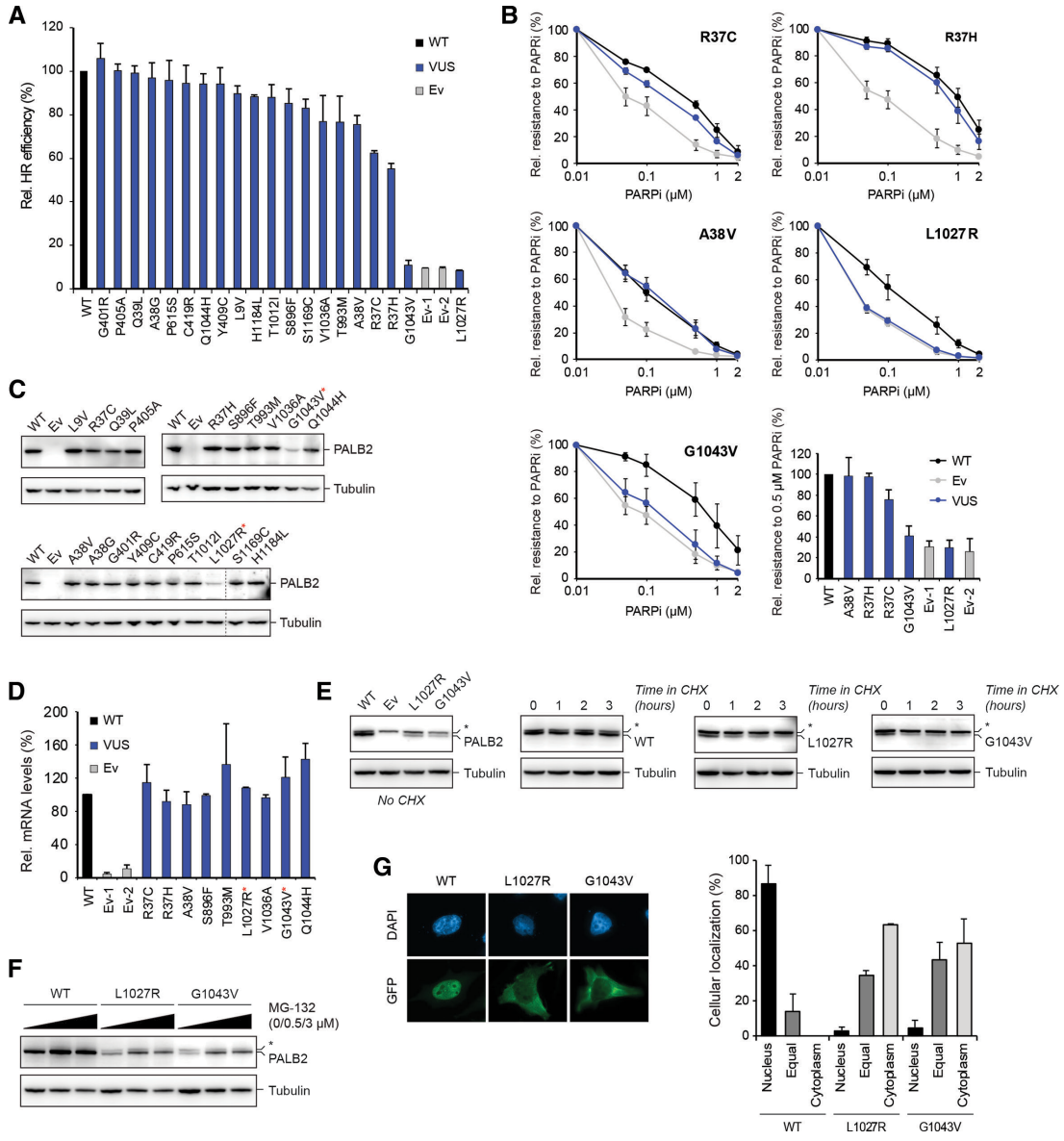

Figure 3 Functional analysis of *PALB2* rare missense variants. (A) HR assay (DR-GFP) in *Trp53*^{KO}/*PALB2*^{KO} mouse embryonic stem (mES) cells complemented with human *PALB2* variants (or an empty vector, Ev). Normalised values are plotted with the wild type (WT) condition set to 100% (absolute HR efficiencies for cells expressing WT *PALB2* were in the range ~7%–10% (adapted from Boonen *et al*[15]). (B) Proliferation-based PARP inhibitor (PARPi) sensitivity assay using mES cells expressing the indicated *PALB2* variants (or an empty vector, Ev). The bar graph showed the relative viability/resistance to 0.5 µM PARPi treatment, for all five variants. (C) Western blot analysis for the expression of all *PALB2* variants analysed. (D) RT-qPCR analysis of selected *PALB2* variants. Primers specific for human *PALB2* cDNA and the mouse PIM1 control locus were used. Tubulin is a loading control. (E) Western blot analysis of PALB2 protein abundance for the indicated variants in the absence of cycloheximide (CHX) and after the indicated time of incubation in the presence of 100 µg/mL CHX. Tubulin is a loading control. Asterisk indicates an aspecific band. (F) Western blot analysis of PALB2 protein abundance for the indicated variants after 24-hour incubation with the indicated concentrations of MG-132. Tubulin is a loading control. Asterisk indicates an aspecific band. (G) Immunofluorescence analysis and quantification for the nucleocytoplasmic distribution of EGFP-PALB2, with or without the indicated variants, following transient expression in HeLa cells. For all bar plots, data represent the mean percentages (±SEM) of the parameter under investigation, with values relative to WT, which was set at 100% (ie, GFP-positive cells (A), viability/resistance (B) and mRNA (D) from at least two independent experiments). Variants/conditions are categorised by colour as either WT (black), VUS (blue) or Ev (grey). Ev1–2 refer to Ev controls from two different replicates. Variants with low expression levels are indicated by *. HR, homologous recombination; PALB2, partner and localiser of BRCA2.

proteasome inhibitor MG-132 further showed that PALB2, with or without the p.L1027R or p.G1043V variant, is subjected to proteasome-dependent degradation (figure 3F). Most likely as a result of protein instability and subsequent proteasomal degradation in the cytoplasm, both the p.L1027R and p.G1043V variants mislocalised in the cytoplasm (figure 3G). These data are concordant with previous localisation data for *PALB2* variants in the WD40 domain, such as p.I944N and p.T1030I, which have also been reported to be unstable and mislocalise in the cytoplasm,[15–17] thereby impacting HR. However, given that several proteins involved in HR, including BRCA2 and RNF168, interact with PALB2's WD40 domain,[1 2 30] we cannot exclude the possibility that these variants also impact HR by affecting the interaction between PALB2 and these proteins.

Overall, the defects for p.L1027R and p.G1043V in HR and PARPi sensitivity are similar to those observed for the Ev

conditions and compare to those previously reported for *PALB2* truncating variants,[15] suggesting they may be similarly pathogenic. Interestingly, the pedigree of the *PALB2* p.L1027R carrier showed that the proband and her maternal aunt were affected by breast cancer at <50 years, and the *PALB2* p.G1043V proband was affected by breast cancer at 55 years. Unfortunately, relatives were not available for predictive testing.

DISCUSSION

Our study confirms that *PALB2* pathogenic variants are associated with an increased breast cancer risk in the South-East Asian population. The estimated prevalence of PTVs (0.73% of patients with breast cancer and 0.14% of controls) is similar to that in European populations,[7] and the estimated OR is also similar to that seen in European populations (OR=4.69 and 5.3).[6 7] However, because the population incidence rates are lower in most populations in South-East Asian than in Western European populations, the absolute risks of *PALB2* carriers are expected to be lower.

To the best of our knowledge, this is the largest study on prevalence of germline *PALB2* variants in a population-based study in South-East Asia. Two case-only studies in the Chinese population, comprising 2769 and 8085 patients with breast cancer, respectively,[31 32] a case-control study of 7051 patients with breast cancer and 11 241 healthy individuals of the Japanese population,[33] and a study of 16 501 breast cancer cases and 5890 healthy Chinese controls[34] have previously been reported. The prevalence of *PALB2* pathogenic variants in our study is consistent with these other Asian studies, which in aggregate reported an average prevalence of 0.74% (range 0.4%–0.97%).

While PTVs in *PALB2* are known to predispose to breast, ovarian and pancreatic cancers, the functional impact of missense variants remains poorly characterised. We found no evidence that rare missense variants, in aggregate, were associated with an increased risk of breast cancer. In addition, we found that none of the in silico measures identified groups of variants which were associated with risk. However, we identified two rare *PALB2* missense variants, both located in WD40 (the critical C-terminus functional domain of PALB2) which were unstable and deficient in HR. Three recent studies on the functional analyses of *PALB2* missense variants revealed that up to 19 deleterious missense variants could abrogate the function of the *PALB2* gene, particularly at the coiled-coil (CC) and the WD40 domains.[15–17] While deleterious variants located in the CC domain have been shown to impair the interaction with BRCA1, deleterious variants located in the WD40 domain often affect protein stability. The identification of two new damaging variants (p. L1027R and p.G1043V) in our study, adds on to the growing lists of *PALB2* variants that could be clinically relevant. Interestingly, the affected carriers with the *PALB2* p.L1027R variants developed early onset breast cancer, suggesting association with breast cancer risk.

This study has some limitations. The Malaysian healthy controls were recruited from women attending opportunistic screening, so there may be enrichment for individuals with higher risk of cancer; indeed 12% of healthy controls reported family history of breast and ovarian cancers, suggesting that this may lead to an underestimate of the risks associated with *PALB2* germline alterations. Some mutations, including large genomic rearrangements and splice variants beyond consensus splice sites, may be missed by the germline amplicon-based panel sequencing method used. However, in *PALB2*, large genomic rearrangements appear to be low relative to small indels or single base substitutions, with most reports failing to identify any such variants.[35–38] It should be noted that for all 20 *PALB2* missense VUS, potential effects on splicing were not examined. Complementation with a bacterial artificial chromosome containing the full-length human gene for *PALB2*, as has recently been shown for *BRCA2*,[39] may allow for the inclusion of splice effects in the future. In addition, despite the size of the study, the number of variants is still low and the confidence limits on the risk estimates are large. In particular, although a clear association with ER-negative and triple-negative breast cancer has been observed in European studies, this was not found in our analysis, perhaps because of limited sample size.

In conclusion, this study has demonstrated that *PALB2* PTVs confer a significant breast cancer risk in the South-East Asian population and that a small proportion of rare missense variants results in loss of function of *PALB2*, which may similarly increase breast cancer risk. These results add to the growing body of evidence of the clinical management of *PALB2* carriers.

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generated sequencing data and performed the bioinformatics analysis; RAB and MS performed the functional assays and analysed results; and SK performed PALB2 localization assays. PSN, RAB, WKH, AA, HvA, DFE and SHT analysed and interpreted the data. PSN and SHT wrote the manuscript which was reviewed and approved by all coauthors.

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