npj | breast cancer

Article

Published in partnership with the Breast Cancer Research Foundation

https://doi.org/10.1038/s41523-025-00848-2

Germline variants of the *POLH* and *RAD51* genes are candidate variants associated with risk of hormone receptor-negative young-onset breast cancer

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Only 15% of young-onset breast cancers have identifiable hereditary germline pathogenic variants (PVs) in an established breast cancer susceptibility gene. However, it is believed that a significant proportion of these breast cancers have additional monogenic or rare risk variants that require identification. To uncover novel cancer susceptibility genes, we performed germline whole-exome/genome sequencing of samples from 564 patients with young-onset breast cancer (aged <40 years), as well as samples from 4032 female controls. The identified candidate variants were further genotyped in 6,967 independent breast cancer cases across all age groups. We identified two PVs that were significantly associated with the risk of hormone receptor-negative young-onset breast cancer: *POLH* p.K589T (OR = 3.65, 95% confidence interval [CI] = 1.28–10.4, $P = 0.0095$) and *RAD51* p.M1fs (OR = 2.15, 95% CI = 1.15–4.02, $P = 0.014$). When *BRCA1/2* PV carriers were excluded from the analysis, only *RAD51* p.M1fs retained a significant association. Whole-genome sequencing of tumor samples carrying these germline risk variants revealed that they harbored mutational signatures indicative of a deficiency of homologous recombination. These findings suggest that hereditary *POLH* p.K589T and *RAD51* p.M1fs are candidate variants associated with an increased risk of hormone receptor-negative breast cancer.

Breast cancer is the most frequently diagnosed cancer among females worldwide, and approximately 30% of cancers in females under the age of 40 are breast cancers[1,2]. In most countries, breast cancer is the primary cause of cancer-related mortality among females. According to the SEER database, 5.6% of all invasive breast cancers are diagnosed in females aged <40. The incidence of invasive breast cancer in young females (aged <40 years) has increased since the early 2000s. Younger age at diagnosis is correlated with high-risk characteristics of breast cancer, such as hormone receptor (HR)-negativity and high-grade histology, as well as with poor prognosis[3]. Young-onset breast cancer is more often familial or hereditary than late-onset breast

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cancer, and 15% of cases of this type of cancer carry germline pathogenic and likely pathogenic variants (PVs) in cancer susceptibility genes[4–6]. For females harboring highly penetrant PVs in genes such as *BRCA1/2* and *TP53*, risk-reducing bilateral mastectomies are an option[7,8]. In carriers of mutations in homologous recombination-associated genes, including *BRCA1/2* and *PALB2*, tumors show a characteristic mutational signature derived from homologous recombination deficiency (HRD) and display sensitivity to poly(ADP-ribose) polymerase (PARP) inhibitors[9]. These findings highlight the importance of genetic testing in young-onset breast cancer to facilitate disease prevention and treatment selection.

In the past decade, many genome-wide association studies have been conducted to identify common variants that affect the risk of developing breast cancer; however, few causal variants have been identified. Genetic linkage and targeted sequencing studies identified loss-of-function (LoF) variants and several rare missense mutations in *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *CHEK2*, *RAD51C*, *RAD51D*, *PALB2*, and *TP53*[10]. The contribution of rare genetic variants to complex traits and diseases has been investigated by whole-exome sequencing (WES) or whole-genome sequencing (WGS), leading to the identification of novel causal variants and genes[11]. However, only approximately half of breast cancers involve common susceptibility variants[12–15]. Thus, young-onset breast cancer, in which genetic predisposition may play an important role, warrants further study. Rare variants that remain unidentified may partly explain the genetic risk in younger patients with breast cancer. It is also unclear whether rare variants reported in Caucasians can also be detected in Asian populations.

Studies of germline variants are generally limited to the assessment of the biological role of germline variants in tumorigenesis, and somatic mutations or mutational signatures are not evaluated. Carriers of PVs associated with high penetrance frequently develop related tumors via second-hit somatic mutations, resulting in biallelic inactivation[16]. These tumors also exhibit somatic and clinical hallmarks of dependence on the germline allele, such as early age of onset and a low number of required somatic oncogenic driver mutations. Integrated germline analysis and somatic tumor profiling are important for assessing the contribution of germline variants to tumorigenesis.

In this study, we performed germline WES or WGS in 564 Japanese females with young-onset breast cancer and 4032 non-cancer controls to identify candidate susceptibility variants. The identified candidate variants were genotyped in an independent cohort of 6,967 breast cancers in all age groups. In addition, WGS was performed on tumor samples with candidate risk variants to determine whether these tumors had genomic characteristics associated with HRD.

Results

Characteristics of study participants

The study included 564 patients (aged <40 years) with young-onset breast cancer who were evaluated by WES or WGS (the young-onset breast cancer cohort), 6967 all-generation patients with breast cancer who underwent TaqMan assays (the all-generation breast cancer cohort), and 4032 Japanese non-cancer controls from the National Cancer Biobank Network (NCBN), all of whom were evaluated by WGS (Fig. 1). The median ages at diagnosis for the young-onset breast cancer cohort (aged <40 years) and all-generation breast cancer cohort were 36 and 56 years, respectively, and 23.4% and 15.4% of the cases were HR-negative, respectively. Patients with a family history of breast and/or ovarian cancer accounted for 19% of the young-onset breast cancer cohort and 4.5% of the all-generations cohort (Table 1).

Germline PVs in 26 known cancer susceptibility genes

First, germline PVs in 26 established cancer susceptibility genes included in the Myriad myRisk Hereditary Cancer Test were evaluated in the young-onset breast cancer cohort. PVs of the 26 cancer susceptibility genes were found in 110 (19.5%) patients (Fig. 2). Among these patients, the median age at diagnosis was 34 years, 67.3% (66/98) were HR-negative, and 34.3% (37/108) had a family history of breast and/or ovarian cancer. PVs of *BRCA 1/2*

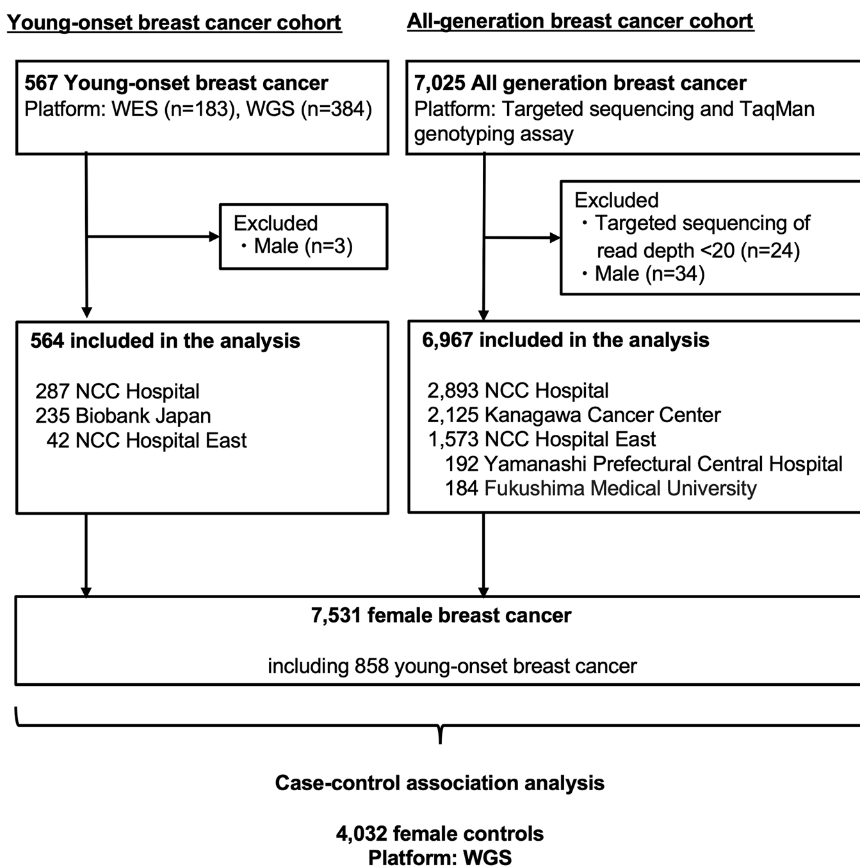


Fig. 1 | STROBE flow chart.

Table 1 | Characteristics of the study participants

Characteristic	N	Cases with breast cancer		P value	Control (n = 4032)
		Young-onset (n = 564)	All-generation (n = 6967)		
Platform		WGS/WES	TaqMan assay		WGS
Age at diagnosis in years, median [range]	7500	36 [20–39]	56 [22–94]	<0.001	65 [16–100][a]
<40 years, n (%)		564 (100)	294 (4.2)		961 (24)
≥40 years, n (%)		0 (0)	6,673 (95.8)		3071 (76)
Histology, n (%)	7255				
Breast carcinoma in situ		36 (6.4)	683 (10)	0.014	—
Invasive ductal carcinoma		437 (77)	4971 (74)		—
Invasive carcinoma, special type		91 (16)	1037 (15)		—
Clinical stage, n (%)	6524				
Stage 0		30 (6.5)	1112 (18)	<0.001	—
Stage I		135 (29)	2035 (34)		—
Stage II		233 (51)	2175 (36)		—
Stage III		48 (10)	468 (7.7)		—
Stage IV		15 (3.3)	273 (4.5)		—
HR status, n (%)	3334				
HR-positive		372 (77)	2407 (85)	<0.001	—
HR-negative		114 (23)	441 (15)		—
HER2 status, n (%)	3235				
HER2-positive		68 (16)	476 (17)	0.8	
HER2-negative		347 (84)	2344 (83)		
Family history of breast or ovarian cancer, n (%)	7528				
In first-degree relatives		72 (13)	273 (3.9)	<0.001	NA
In first- and/or second-degree relatives		107 (19)	314 (4.5)		NA

HER2 human epidermal growth factor receptor 2, *HR* hormone receptor, *NA* not available, *WGS* whole-genome sequencing, *WES* whole-exome sequencing.
[a]Age at sample collection.
Number (%) of missing data (Young-onset/All-generation): Age 0/31 (0.4), Histology 0/276 (4), Stage 103 (18)/904 (13), HR 78 (14)/4119 (59), HER2 149 (26)/4147 (60), Family history 0/3 (0.04).

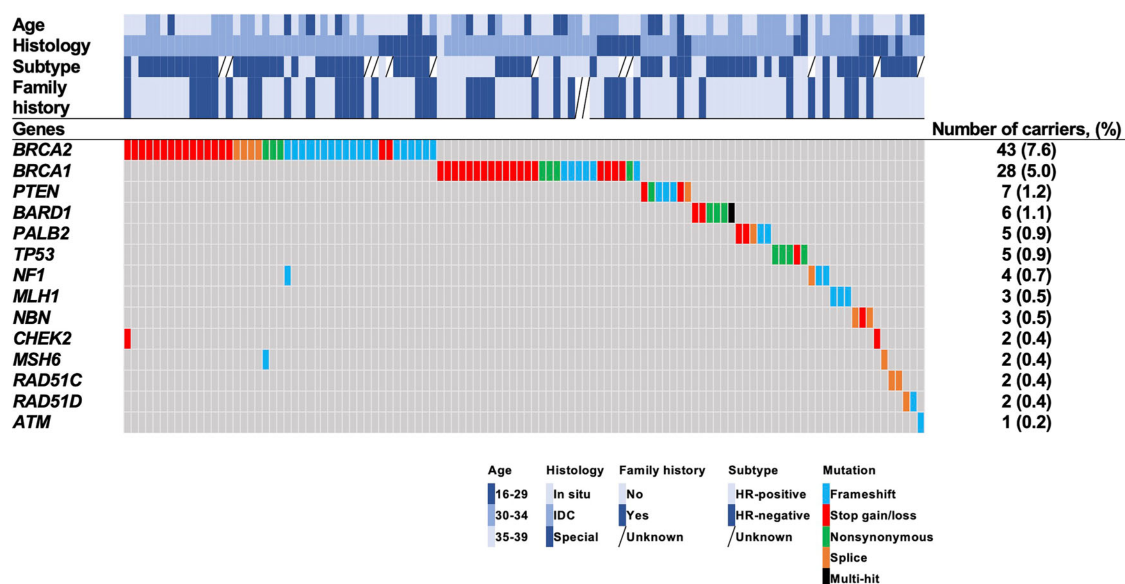


Fig. 2 | Germline variants in 26 cancer susceptibility genes in young-onset breast cancer. The 113 germline pathogenic variants were identified in 110 young-onset breast cancer patients. Each column corresponds to a patient. The upper panel shows the patient's clinical characteristics. IDC invasive ductal carcinoma, HR hormone receptor.

(n = 71, 12.6%), *PTEN* (n = 7, 1.2%), *BARD1* (n = 6, 1.1%), *PALB2* (n = 5, 0.9%), and *TP53* (n = 5, 0.9%) were frequently observed. These PVs were mutually exclusive, whereas three patients had two PVs, with *CHEK2*, *NF1*, and *MSH6* overlapping with the *BRCA2* PVs.

Identification of novel risk variants in young-onset breast cancers

To identify novel candidate risk variants, we evaluated germline variants of 692 genes associated with cancer susceptibility and/or with DNA repair

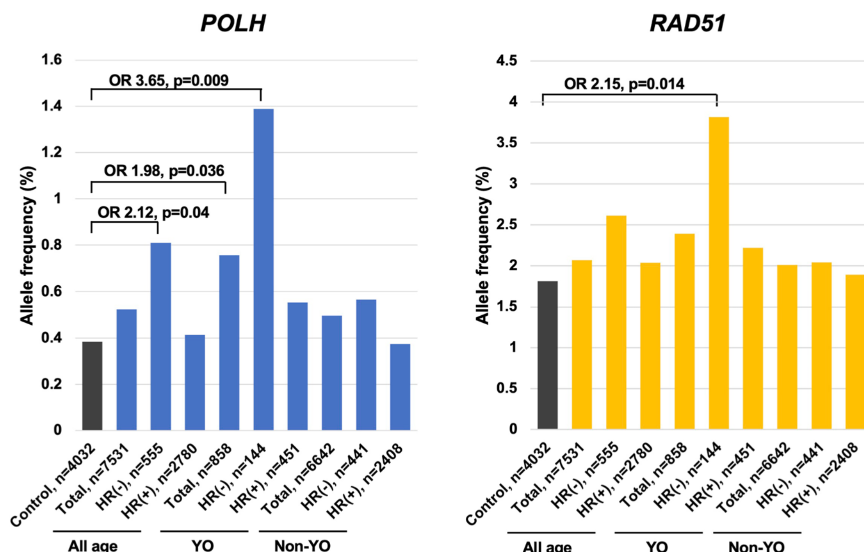


Fig. 3 | Differences in allele frequency of *POLH* and *RAD51* variants by hormone receptor status and age. Allele frequencies of the *POLH* and *RAD51* variants in the case and control groups are shown. *POLH* p.K589T and *RAD51* p.M1fs variants were significantly associated with HR-negative young-onset breast cancers (*POLH*: OR = 3.65, 95% CI = 1.28–10.4, *P* = .0095; *RAD51*: OR = 2.15, 95% CI = 1.15–4.02, *P* = .014). HR hormone receptor, YO young-onset.

functions based on previous reports (Supplementary Data S1)[17–20]. According to our selection criteria, we identified one pathogenic missense and two LoF variants that were possibly associated with the risk of HR-negative young-onset breast cancer (Supplementary Fig. 1). The first risk variant was a pathogenic missense variant, p.K589T (rs121908565), in *POLH* (polymerase eta), observed in 3 of 114 HR-negative young-onset breast cancers and 31 of 4,032 NCBN controls (2.6% vs. 0.77%, OR = 3.46, 95% CI = 1.05–11.4, *P* = .03). The second risk variant was a frameshift variant, p.74Sfs*8 (rs541992483) in *BPIFB4* (BPI fold-containing family B, member 4). This variant was found in 3 of 114 HR-negative young-onset breast cancers and 32 of 4032 controls (2.6% vs. 0.79%, OR = 3.35, 95% CI = 1.02–11.01, *P* = .035). The third variant was the frameshift variant pM1fs (rs55714242) in *RAD51* (RAD51 recombinase), found in 10 of 114 HR-negative young-onset breast cancers and 146 of 4,032 controls (8.8% vs. 3.6%, OR = 2.49, 95% CI = 1.29–4.79, *P* = .0048) (Supplementary Data S2 **(A)**). Forty-two patients with young-onset breast cancer carried one of the three candidate risk variants, and their median age at diagnosis was 37 years. Sixteen (39%, 16/41) were HR-negative, and five (12%, 5/41) had a family history of breast and/or ovarian cancer. Eight patients (19%, 8/42) also carried germline PVs in *BRCA1*, *BRCA2*, and *MLH1* (Supplementary Fig. 2). To investigate the mutual exclusion between the candidate variants and known high-penetrance PVs such as *BRCA1/2*, we performed an analysis in which patients with *BRCA1/2* PV were excluded. The estimated OR of candidate variants decreased, and only *RAD51* pM1fs was associated with HR-negative breast cancers (Supplementary Data S2 **(B)**). Although genograms were not available for carriers with these risk variants in the young-onset breast cancer cohort, our review of patients referred to the National Cancer Center Hospital for genetic counseling who underwent germline WGS identified a 38-year-old *POLH* p.K589T carrier with *BRCA1/2*-negative breast cancer who had a family history of breast and pancreatic cancer (Supplementary Fig. 3).

Frequency of rare coding variants identified in the European population

Recently, whole-exome-based association studies have identified many novel rare coding variants associated with breast cancer risk in persons of European ancestry[11]. We examined the frequency of PVs in the 12 genes identified in this study (*MAP3K1*, *LZTR1*, *MMP26*, *ATRIP*, *BAP1*, *KCND2*, *CUL9*, *CFAP126*, *GPR37*, *TGM7*, *ZFYVE19*, and *SEC62)* in our cohort. However, only two patients with young-onset breast cancer had PVs in *LZTR1*, and no PVs were detected in any of the other genes (Supplementary Data S3).

Validation of candidate variants across all-age breast cancer cases

To further assess age-specific associations between candidate variants and breast cancer, we genotyped *POLH* and *RAD51* variants in 6,967 patients with breast cancer across all age groups. The *BPIFB4* variant was not genotyped because the corresponding TaqMan PCR assay could not be designed. Compared with the same 4,032 controls described above, *POLH* p.K589T and *RAD51* p.M1fs variants were significantly associated with HR-negative young-onset breast cancers (*POLH*: OR = 3.65, 95% CI = 1.28–10.4, *P* = .0095; *RAD51*: OR = 2.15, 95% CI = 1.15–4.02, *P* = .014), but not with HR-negative non-young-onset breast cancers. Additionally, *POLH* p.K589T also showed nominal associations with young-onset breast cancers irrespective of HR status (OR = 1.98, 95% CI = 1.03–3.79, *P* = .036), and with HR-negative breast cancers regardless of age (OR = 2.12, 95% CI = 1.01–4.46, *P* = .04; Fig. 3, Supplementary Data S4).

Tumor characteristics of POLH and RAD51-mutated breast cancers

We next evaluated the whole-genome landscape of tumor samples with germline risk variants of *POLH* and *RAD51* to determine whether these tumors exhibited HRD-associated mutational signatures. Among the 43 females with breast cancers harboring *POLH* (n = 10) or *RAD51* (n = 33) germline risk variants for which fresh frozen tumor tissues were available, 12 patients (four *POLH* and eight *RAD51*) with triple-negative breast cancer (TNBC, estrogen and progesterone receptor <10%) or young-onset disease were selected for WGS analysis. Two patients, one with a *POLH*-variant and another with a *RAD51*-variant, had concurrent germline or somatic LoF mutations in *BRCA1*. All three *POLH*-mutated breast cancers without concurrent *BRCA1* mutations were classified as HRD using the HRDetect and CHORD algorithms (Fig. 4). One of the three *POLH*-mutated breast cancers showed loss of heterozygosity (LOH) at the variant locus. Of the seven *RAD51*-mutated breast cancers without concurrent *BRCA1* mutations, one displayed LOH and was classified as having HRD. Circos plots of the genomic structures of representative HRD cases with *POLH* or *RAD51* variants are shown in Fig. 5. A *POLH*-mutated TNBC with predicted *BRCA1*-type HRD has a large number of tandem duplications, whereas a *RAD51*-mutated TNBC with predicted *BRCA2*-type HRD has a large number of deletions. Finally, we examined the presence of LOH in additional tumors from carriers of *POLH* (n = 21) or *RAD51* (n = 87) variants. LOH was found in six (28.5%) and four (4.6%) tumors from patients with *POLH* or *RAD51* variants, respectively (Supplementary Data S5).

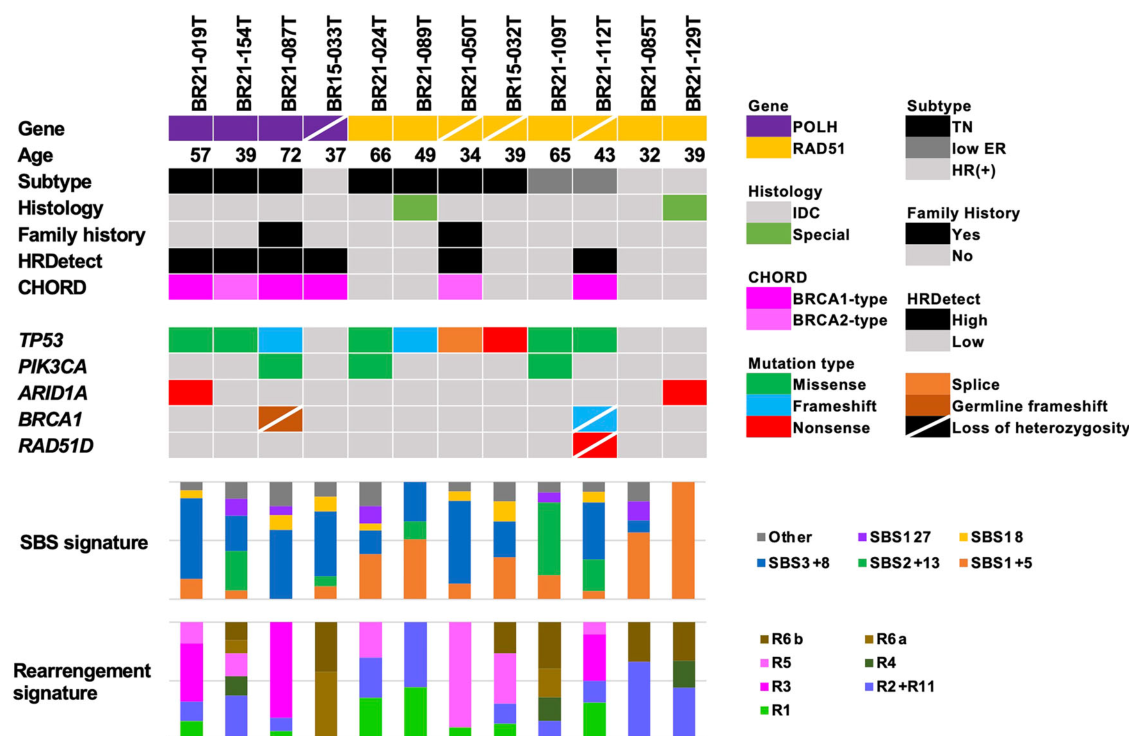


Fig. 4 | Genomic landscape of *POLH* and *RAD51* mutated breast tumors. Oncogenic or pathogenic somatic mutations, and mutational signatures identified in 12 tumors from patients carrying *POLH* (n = 4) or *RAD51* (n = 8) variants. The phenobar provides information on clinicopathological features, HRDetect, and CHORD. SBS single base substitution, IDC invasive ductal carcinoma, HR hormone receptor, TN triplenegative, ER estrogen-receptor, HG histological grade.

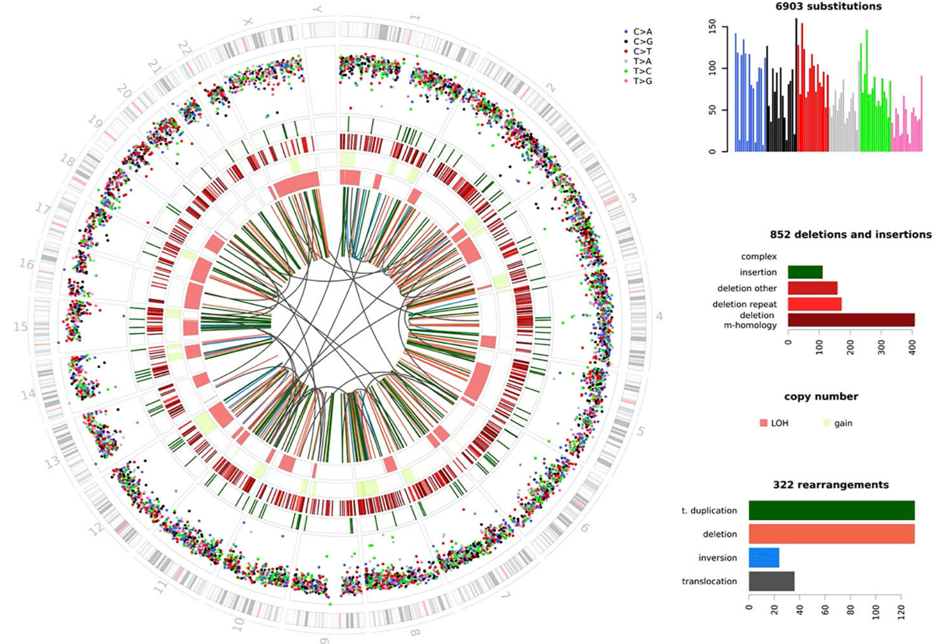
Discussion

Breast cancers diagnosed in young females are more often familial or hereditary than in older females, but the causative genes are unknown in most cases. In this study, we found that germline variants in *POLH* and *RAD51* moderately increased the risk of young-onset HR-negative breast cancer.

Young-onset breast cancer is associated with a high frequency of germline PVs. PVs in *BRCA1/2* are the most frequent, accounting for 10–14% of young-onset breast cancer regardless of ethnicity[6,21,22]. In this study, 12.6% of the young-onset breast cancer cohort had *BRCA1/2* PVs, which is consistent with the results of previous reports. When the analysis was expanded to 26 known cancer susceptibility genes, 19.5% had germline PVs, which is also consistent with a study of 35,000 cases in a multi-ethnic population (13–18%)[4]. However, among rare coding variants other than those of known breast cancer susceptibility genes identified in European populations, only two PVs in *LZTR1* were found in Japanese young-onset breast cancer. These results suggest that the contribution of low-to-moderate breast cancer susceptibility genes to young-onset breast cancer may differ according to ethnicity.

RAD51 is a well-established central protein in DNA double-strand break repair and is regulated by several proteins, including BRCA2, PALB2, and RAD51 paralogs[23]. Following double-strand breaks, RAD51 binds to DNA and forms a nucleoprotein filament that invades the homologous double helix. RAD51 paralogs, including RAD51C and RAD51D, contribute to the stabilization and elongation of RAD51 filaments[24]. Although *RAD51C* and *RAD51D* are established susceptibility genes for breast cancer, the impact of *RAD51* on breast cancer risk remains to be elucidated. Although the single-nucleotide polymorphism −135 G > C in *RAD51* was reported to be associated with an increased risk of breast cancer in *BRCA2* carriers[25], no large case-control or genome-wide association study has demonstrated that *RAD51* is a breast cancer susceptibility gene in the general population[26–29]. By contrast, few studies have evaluated the contribution of *POLH* to breast cancer risk. *POLH* is involved in DNA damage repair via its translesion synthesis activity and is a causative gene for xeroderma pigmentosum (XP) variant disease. XP is associated with an increased risk of skin cancers, central nervous system tumors, hematologic malignancies, and gynecological cancers, but not breast cancers[30]. In the GENESIS study that examined 113 DNA repair-related genes in 1,207 French patients with *BRCA1/2*-negative familial breast cancer and 1,199 controls, *POLH* PVs were not associated with an increased risk of breast cancer[31,32]. Given that allele frequencies of *POLH* and *RAD51* are highest in East Asian populations according to the gnomAD database, the contribution of *POLH* and *RAD51* to breast cancer risk may be specific to these populations.

In addition to providing evidence that *POLH* and *RAD51* variants are associated with breast cancer risk, we observed that tumors harboring *POLH* variants exhibited genomic characteristics consistent with HRD. POLH contributes to homologous recombination by interacting with BRCA2 and PALB2 to form a D loop[33,34]. Our findings from WGS analysis of tumors suggest that *POLH* variants play a critical role in tumorigenesis. In the latest ClinVar, *POLH* p.K589T has been reclassified as a variant of uncertain significance. However, others classified this variant as pathogenic because it was originally identified in a Japanese family with XP variant disease, and cells with the *POLH* p.K589T mutation display decreased recovery of DNA synthesis after irradiation[35]. Furthermore, the gene expression level of *POLH* in breast-mammary tissue was 6.99 TPM in the GTEx database (Supplementary Data S2), supporting the idea that decreased *POLH* gene expression by the LoF variant may have a high functional impact. Interestingly, LOH was found in only one-quarter of patients with *POLH* variants and HRD, indicating that a single-allele variant may contribute to carcinogenesis, similar to *POLE*[36]. By contrast, the HRD phenotype was observed in tumors from *RAD51* variant carriers only in the presence of LOH. Although the *RAD51* p.M1fs variant is annotated as likely benign in ClinVar primarily because of its relatively high allele frequency in the general population, it causes a frameshift at the initiation codon and eliminates the canonical start site, likely resulting in a LoF effect on the RAD51 protein.



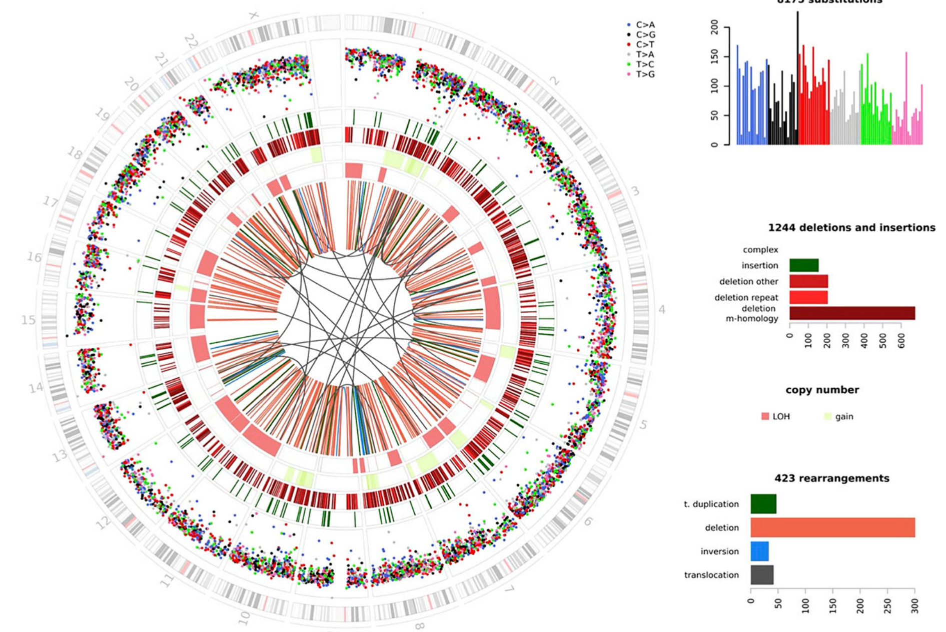


Fig. 5 | Circos plots of *POLH* and *RAD51*-mutated breast cancers. Circos plots of *POLH* (upper panel) and *RAD51* (lower panel) mutated breast cancers are shown. The first outer circle represents the chromosomes. The second circle shows the base substitutions. Circles with short green linings represent insertions; circles with short red lines represent deletions. The third circle shows the major copy number changes (green, gain). The fourth circle represents the minor allele copy number (red, loss). The central lines represent rearrangements.

Given the unfavorable prognosis associated with young-onset breast cancer and the limited therapeutic options available for HR-negative breast cancer, there is an urgent need to develop early cancer detection methods and novel treatment strategies. The present study identified *POLH* p.K589T and *RAD51* p.M1fs variants in 1.5% and 4.8% of Japanese young-onset breast cancers, respectively. However, *POLH* is currently not included in widely used gene panel tests, and identifying *POLH* variants is thus difficult.

This study had several limitations. First, although germline whole exome/genome analysis was performed, we focused on the 692 selected genes. This approach may have underestimated the presence of potential candidate risk variants. Second, information on breast cancer subtypes was

unavailable for a significant number of cases. Third, it remains unclear whether the *POLH* variant functions as a modifier that enhances breast cancer risk in the presence of high-penetrance PVs or as an independent low- to moderate-risk variant. Given that the allele frequency of the *POLH* variant was less than 1%, the statistical power of the subgroup analysis was limited. Further investigations are necessary to address this question. Lastly, this study lacked an independent cohort to evaluate the association between candidate variants and the risk of young-onset breast cancer. However, young-onset breast cancer is a rare occurrence, accounting for approximately 5% of all breast cancers. Consequently, this study represents one of the largest case-control studies conducted on homogenous racial populations, and it thus provides valuable insight into this particular issue.

In conclusion, PVs in *POLH* and *RAD51* may contribute to susceptibility to HR-negative young-onset breast cancer. If further large-scale studies confirm their association with the risk of young-onset breast cancer, these genes should be incorporated into gene panel tests to predict heritable risk.

Methods

Study cohorts

The young-onset breast cancer cohort comprised 567 patients diagnosed with breast cancer aged <40 years who were collected from three institutions: National Cancer Center (NCC) Hospital, NCC Hospital East, and Biobank Japan. Biobank Japan is a multi-institutional, hospital-based registry that collects DNA and clinical information from patients with various common diseases, including breast cancer, from all over Japan[37,38]. The all-generation breast cancer cohort included 7,025 patients with breast cancer from all age groups from five institutions: NCC Hospital, NCC Hospital East, Kanagawa Cancer Center, Yamanashi Prefectural Center Hospital, and Fukushima Medical University. After excluding males with breast cancer and those who did not have sufficient sequence depth for targeted sequencing[6,39], 7,531 female cases (including 858 young-onset breast cancer cases) were analyzed. The controls were recruited from the NCBN[40,41], and comprised 4,032 females aged ≥16 years, none of whom had a history of cancer. A STROBE flow chart of the study is shown in Fig. 1.

Sequencing of germline variants

Genomic DNA was extracted from leukocytes and non-cancerous breast cancer tissues using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) or the Allprep DNA Mini Kit (Qiagen, Hilden, Germany). For 183 breast cancer patients, WES was performed using the Agilent SureSelect Human All Exon V4 or V5 platform and the Illumina Nextera Exome Kit and Nextera DNA Library Prep for Enrichment according to the manufacturer's instructions. Sequencing of the 75 bp paired-end reads was performed using HiSeq2500 (Illumina, San Diego, CA, USA) at a depth of approximately 100×. For 384 breast cancer patients, WGS libraries were prepared using the TruSeq DNA PCR-Free Library Prep Kit (Illumina). Sequencing of the 150 bp paired-end reads was performed using NovaSeq6000 (Illumina) at a depth of approximately 30×. FASTQ data from 235 cases in Biobank, Japan, were downloaded from NBCD (JGAS000114). The resulting FASTQ data were subjected to genome mapping and variant calling using our in-house data analysis pipeline. Genome mapping was performed using Parabricks v3.1.3 (NVIDIA), which delivers the high-speed analysis recommended by the Genomic Analysis Toolkit (GATK) with GPU acceleration[42]. Genome Reference Consortium Human Build 38 was used as the reference sequence. The pipeline used in this study implemented algorithms equivalent to those of Burrows–Wheeler Aligner (v0.7.15)[43] and GATK (v4.1.0). Duplicates from mapped reads were flagged, and realignment and base quality score recalibration were performed. Mapped data were outputed in BAM format[44]. Variant calls were converted into the gVCF format for joint calling. Genotyping of candidate variants was performed using GATK HaplotypeCaller[45], with the hard-filtering setting suggested by GATK.

Germline variant classification

Two gene sets were evaluated, namely 26 established cancer susceptibility genes (*APC, ATM, BARD1, BMPR1A, BRCA1, BRCA2, BRIP1, CDK4, CDKN2A, CDH1, CHEK2, EPCAM, NBN, NF1, MLH1, MSH2, MSH6, MUTYH, PALB2, PMS2, PTEN, RAD51C, RAD51D, SMAD4, STK11*, and *TP53*) included in the Myriad myRisk Hereditary Cancer Test[4], and 692 genes associated with cancer predisposition and DNA repair reported in previous studies[17–20] (**Supplemental Data S1**). Germline variants were considered pathogenic if they met the following criteria [(1) + (2) + (3) or (1) + (2) + (4) or (1) + (2) + (5)]: (1) global minor allele frequency (MAF) < 0.05 in ExAC[46] and/or Tohoku Medical Megabank Organization[47]; (2) variant allele frequency (VAF) ≥30% and ≤70%; (3) null variants (nonsense, frameshift indel, and splice-site variants) and missense variants classified as "pathogenic" or "likely pathogenic" in ClinVar[48] (https://www.ncbi.nlm.nih.gov/clinvar/); (4) high-impact LoF variants such as stop-gain, stop-loss, start-loss, frameshift, splice acceptor gain or loss, and splice donor gain or loss defined by SnpEff v4.3[49]; and (5) splice variants with a delta score >0.5 annotated by SpliceAI[50]. Finally, annotations of each variant were reviewed by an expert panel. Pathogenic variants were validated by Sanger Sequencing[51].

Selection of candidate susceptibility genes and risk variants in young-onset breast cancers

Candidate risk variants were selected according to the following criteria: (1) variants classified as pathogenic according to the criteria listed above; (2) variants detected in ≥5 cases; and (3) variants with odds ratio (OR) ≥1.5 and *P* value < 0.05 against NCBN female controls. The selected candidate risk variants in the all-generation breast cancer cohort were further genotyped using TaqMan SNP Genotyping Assays.

Detection of genomic alterations by WGS

DNA was extracted from the tumor and matched normal tissues and subjected to library preparation. Tumor sequencing was performed using NovaSeq6000 (Illumina) at an approximate depth of 120×, whereas matched normal tissue sequencing was performed at an approximate depth of 30×. The resulting reads were aligned to the hg38. Somatic SNVs were called using mutect2 (gatk version 4.1.2.0)[52], and small indels were called using mutect2 and strelka2[53]. The detected variants were annotated using OncoKB[54], ClinVar[48], and SnpEff[49], and oncogenic variants were defined as those annotated as oncogenic or likely oncogenic in the OncoKB database[54]. These variants were validated using the Integrative Genomics Viewer (IGV)[55]. Single base substitution signatures were estimated using the Fit Multi-Step (FitMS) algorithm introduced in previous studies[56,57]. Breast-specific signatures detected in the Genomics England (GEL) cohort were assigned to each sample. Structural variants were detected using Manta (version 1.6.0)[58], and rearrangement signatures were extracted from Signature.tools.lib[59]. SVs were classified into 32 SV types based on size, topology, and junction clustering as previously described, and were fit to 20 rearrangement signatures derived from 3,107 cancers[57]. Allele-specific copy number, tumor purity, and ploidy were estimated by facets (version 0.6.2)[60]. Loss of heterozygosity (LOH) was considered to be present when the total copy number of a gene was one and the minor copy number was zero.

HRD prediction

HRD status was predicted by R package Classifier of HOmologous Recombination v2.0 (CHORD)[61] and HRDetect[59], as previously described. HRD status by CHORD and HRDetect was determined as HRD when the predictive score was >0.5 and >0.7, respectively. CHORD also distinguished *BRCA1*-type or *BRCA2*-type HRD based on 1–100 kb duplications.

Detection of copy number alterations using the TaqMan assay

Copy number alterations were detected by real-time genomic PCR using the TaqMan copy number assay and ABI 7900HT real-time PCR system (Thermo Fisher Scientific, MA, USA). The two genes, *POLH*

(NM_006502.3) and *RAD51* (NM_002875.5), and all TaqMan probes, including *POLH* (ID Hs00165713_cn), *RAD51* (ID Hs00114987_cn), and *RNase P* (cat. no. 4403328), were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Genomic data were analyzed using the ABI PRISM 7900HT Sequence Detection Software CopyCaller v2.1 (Thermo Fisher Scientific) for copy number analysis. LOH was determined by CopyCaller according to the manufacturer's instructions.

Statistical analysis

The Mann-Whitney U test was used for continuous variables, and Fisher's exact test was used for categorical variables. Case-control association analyses were performed using Fisher's exact test to calculate the OR and 95% confidence interval (CI) for each variant. In addition, PLINK1.06 was used for the statistical analysis of association studies. All tests were two-tailed and the significance level was set at $\alpha = .05$. The Bonferroni correction was applied for association analysis between the two genotyped variants and breast cancer ($P < .025 = 0.05/2$). Association analyses were prespecified for the overall cohort and for subgroups defined by age (≥40 vs. <40 years) and HR status (positive vs. negative). Statistical analyses were performed using STATA (version 15.1; StataCorp, College Station, TX, USA) and GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

Ethics approval

This study was approved by the Institutional Review Board of all participating institutions: NCC Hospital (2015-278, 2017-353, and 2019-229), NCC Hospital East (2015-278 and 2017-353), Kanagawa Cancer Center (2017-74), Yamanashi Prefectural Center Hospital (1709-28), Fukushima Medical University (29275). All patients provided written informed consent to participate. This study involving human material and data has been performed in accordance with the Declaration of Helsinki.

Data availability

Genome data are available from the National Bioscience Database Center (NBDC) Human Database (research ID: JGAS000114). Other genome data that support the findings of this study and further information are available from the corresponding author upon reasonable request.

Code availability

The code used for the data analysis presented in this manuscript utilizes publicly available software packages with no customization. The code can be provided upon reasonable request.

Received: 22 May 2025; Accepted: 23 October 2025;
Published online: 28 November 2025

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Acknowledgements

The authors wish to thank physicians and staff members at the National Cancer Center and other hospitals for their assistance and support. We thank all of the subjects for participating in the study, as well as Maiko Matsuda, Yoko Shimada, Sadaaki Takata, Hiromichi Nakajima, Hikari Kiyohara, Akira Hirota, Mao Uematsu, Misao Fukuda, and Nobuyuki Takahashi for assisting with sample collection. We acknowledge the staff of the Laboratory for Genotyping Development in RIKEN, the RIKEN-IMS Genome Platform, the NCBN Controls WGS Consortium, and the BioBank Japan project. We would like to express our gratitude to Bioedit for assisting with English language editing. This research was supported in part by Japan Agency for Medical Research and Development (AMED) (JP15ck0106096 and 25ck0106879h0003 to T.Ko., 19cm0106605h0003 and 23ama221520h0001 to K.Sh., and JP19kk0305010 to Y.Mo.), Health Labour Sciences Research Grant (202108001B to K.Sh.), the Japan Society for the Promotion of Science (JSPS) KAKENHI Early-Career Scientists JP18K16292 (to Y.H.), a Grant-in-Aid for Scientific Research (B) 20H03668 and 23H02955 (to Y.H.), 17H06162 (to H.N.), 20H03695 (to K.Sh.), and 16H06277 (CoBiA: to Y.Mo.), a Grant-in-Aid for the Genome Research Project from Yamanashi Prefecture (to M.O. and Y.H.), BRIDGE (programs for bridging the gap between R&D and the ideal society (Society 5.0) and generating economic and social value to K.Sh.), National Cancer Center Research, and Development Fund (2022-A-20, 2023-J-2, NCC Biobank, and NCC Core Facility to K.Sh.).

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S.Y., T.Ko., and K.Sh. conceptualized the study and developed the methodology. S.Y., R.K., Y.Mo., T.Yo., T.Ya., S.S., C.Y., K.H., M.S., Y.H., H.A., R.H., C.S., A.Sh., T.S., K.Su., M.Y., K.Sun., M.Hi., Y.Y., T.Kog., T.Mu., S.F., Y.Mi., K.Ta., K.M., Y.Mur., H.N., K.To.,Y.K., NCBN Controls WGS Consortium, Biobank Japan Project, M.O., T.Oh., A.Su., T.On., Y.N., T.Yam., K.Y., T.Ko., and K.Sh. provided patient samples for the study. Y.Mo., S.T., A.O., Y.Shim., and K.Sh. performed the experiments. S.Y., R.K., and H.A. curated the data. Y.Shir., M.T., A.M., K.Hi., E.F., K.K., M.Ho., and A.K. supervised the analyses and contributed to data interpretation. S.Y. and K.Sh. performed the formal analysis. S.Y. and K.Sh. wrote the original draft of the manuscript. All authors reviewed and edited the manuscript.

Competing interests

Masayuki Yoshida reported receiving personal fees from Roche Japan, Agilent Technologies, Chugai Pharma, Ono Yakuhin, MSD, and Daiichi Sankyo. He has participated on a Data Safety Monitoring Board or Advisory Board for Daiichi Sankyo. Kenichi Harano reported receiving grants from AstraZeneca, Chugai, Daiichi Sankyo, MSD, and Takeda, and personal fees from AstraZeneca, Chugai, Eisai, MSD, Taiho, and Takeda. He has participated on a Data Safety Monitoring Board or Advisory Board for AstraZeneca, Chugai, Daiichi Sankyo, Taiho, and Takeda. Takashi Yamanaka reported receiving personal fees from Daiichi Sankyo, Eli Lilly Japan, AstraZeneca, Chugai, Pfizer Japan, Kyowa Kirin, and Taiho. Kazunoshin Tachibana reported receiving grants from Chugai, Eisai, Taiho, Takeda, MSD, Daiichi Sankyo, Eli Lilly, Asahi Kasei, Nihon Kayaku, Kyowa Kirin, Astellas, and Maruho, and personal fees from Chugai, AstraZeneca, Pfizer, Eisai, Daiichi Sankyo, Eli Lilly, MSD, Kyowa Kirin, Teijin, Taiho, PDR Pharma, and Exact Sciences. Chikako Shimizu reported receiving personal fees from Chugai and has participated on a Data Safety Monitoring Board or Advisory Board for Daiichi Sankyo. Akihiko Shimomura reported receiving grants from Chugai Pharmaceutical, AstraZeneca, and Eisai, and personal fees from AstraZeneca, Daiichi Sankyo, Pfizer, Eli Lilly, MSD, Chugai Pharmaceutical, Nihon Medi-Physics, Taiho Pharmaceutical, and Exact Sciences. Takahiro Kogawa reported receiving grants from Eli Lilly, AstraZeneca, and Guardant Health; consulting fees from Daiichi Sankyo and Astellas Pharma; and personal fees from Daiichi Sankyo, Ono Pharma, Gilead Sciences, Astellas Pharma, Eisai, AstraZeneca, Taiho Pharma, and Chugai Pharma. He has received payment for expert testimony from Astellas Pharma and support for attending meetings from Pfizer and Eisai. He has participated on a Data Safety Monitoring Board or Advisory Board for Daiichi Sankyo, Ono Pharma, Gilead Sciences, Oncotherapy Sciences, Eisai, AstraZeneca, and Taiho Pharma. Tohru Ohtake reported receiving grants from Chugai, Eisai, Taiho, Takeda, Asahi Kasei, Daiichi Sankyo, Eli Lilly, Nihon Kayaku, and Kyowa Kirin, and personal fees from Chugai, Pfizer, AstraZeneca, Eisai, Daiichi Sankyo, Eli Lilly, Kyowa Kirin, Novartis, FUJIFILM Toyama Chemical, Johnson & Johnson, Asahi Kasei, Exact Sciences, Otsuka, and MSD. Tatsuya Onishi reported receiving grants from Daiichi Sankyo and Bayer Pharma, and personal fees from Daiichi Sankyo. Toshinari Yamashita reported receiving grants from Chugai, Taiho, Nippon Kayaku, Eli Lilly, Daiichi Sankyo, Pfizer, AstraZeneca, Seagen, MSD, Kyowa Kirin, Ono, Gilead Sciences, and Eisai, and personal fees from Chugai, Eisai, Daiichi Sankyo, Taiho, Nippon Kayaku, AstraZeneca, Kyowa Kirin, Pfizer, Eli Lilly, Novartis Pharma, and MSD. Yoichi Naito reported receiving grants from AbbVie, Ono, Daiichi Sankyo, Taiho, Pfizer, Boehringer Ingelheim, Eli Lilly, Eisai, AstraZeneca, Chugai, and Bayer, and personal fees from AstraZeneca, Eisai, Ono, Guardant, Takeda, Eli Lilly, Novartis, Pfizer, Chugai, PDR Pharma, Nihon Kayaku, Taiho, Bristol Myers Squibb, Bayer, Daiichi Sankyo, and MSD. Kan Yonemori reported receiving grants from MSD, Daiichi Sankyo, Merck Biopharma, AstraZeneca, Taiho, Pfizer, Novartis, Takeda, Chugai, Ono, Sanofi, Seagen, Eisai, Eli Lilly, Genmab, Boehringer Ingelheim, Kyowa Hakko Kirin, Nihon Kayaku, and Haihe; personal fees from Pfizer, Eisai, AstraZeneca, Eli Lilly, Takeda, Chugai, Fuji Film Pharma, PDR Pharma, MSD, Boehringer Ingelheim, Ono, Daiichi Sankyo, Bayer, Janssen, Astellas, Bristol Myers Squibb, Novartis, and Sanofi; and has participated on a Data Safety Monitoring Board or Advisory Board for Eisai, AstraZeneca, Sanofi, Genmab, Gilead, OncXerna, Takeda, Novartis, MSD, and Henlius. All remaining authors declare no conflicts of interest.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41523-025-00848-2.

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