JNCI: Journal of the National Cancer Institute, 2024, 116(1), 149–159
https://doi.org/10.1093/jnci/djad183
Advance Access Publication Date: September 9, 2023
Article

Germline pathogenic variants in neuroblastoma patients are enriched in *BARD1* and predict worse survival

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Abstract

Background: Neuroblastoma is an embryonal cancer of the developing sympathetic nervous system. The genetic contribution of rare pathogenic or likely pathogenic germline variants in patients without a family history remains unclear.

Methods: Germline DNA sequencing was performed on 786 neuroblastoma patients. The frequency of rare cancer predisposition gene pathogenic or likely pathogenic variants in patients was compared with 2 cancer-free control cohorts. Matched tumor DNA sequencing was evaluated for second hits, and germline DNA array data from 5585 neuroblastoma patients and 23 505 cancer-free control children were analyzed to identify rare germline copy number variants. Patients with germline pathogenic or likely pathogenic variants were compared with those without to test for association with clinical characteristics, tumor features, and survival.

Results: We observed 116 pathogenic or likely pathogenic variants involving 13.9% (109 of 786) of neuroblastoma patients, representing a statistically significant excess burden compared with cancer-free participants (odds ratio [OR] = 1.60, 95% confidence interval [CI] = 1.27 to 2.00). *BARD1* harbored the most statistically significant enrichment of pathogenic or likely pathogenic variants (OR = 32.30, 95% CI = 6.44 to 310.35). Rare germline copy number variants disrupting *BARD1* were identified in patients but absent in cancer-free participants (OR = 29.47, 95% CI = 1.52 to 570.70). Patients harboring a germline pathogenic or likely pathogenic variant had a worse overall survival compared with those without ($P = 8.6 \times 10^{-3}$).

Conclusions: *BARD1* is an important neuroblastoma predisposition gene harboring both common and rare germline pathogenic or likely pathogenic variations. The presence of any germline pathogenic or likely pathogenic variant in a cancer predisposition gene was independently predictive of worse overall survival. As centers move toward paired tumor-normal sequencing at diagnosis, efforts should be made to centralize data and provide an infrastructure to support cooperative longitudinal prospective studies of germline pathogenic variation.

Neuroblastoma is an embryonal malignancy of early childhood that arises from developing postganglionic sympathetic neurons and accounts for 12% of all childhood cancer-related deaths (1). Patients are classified into low, intermediate, and high risk based on a series of clinical and tumor biological features, and this risk group is used for treatment stratification purposes (1). Despite

Received: March 09, 2023. **Revised:** August 02, 2023. **Accepted:** August 25, 2023

aggressive multimodal therapy, nearly 50% of high-risk neuroblastoma patients diagnosed at older than 18 months of age eventually succumb to their disease. A subset of these tumors harbor somatic *MYCN* amplification and/or an activating somatic *ALK* mutation or gene amplification (1). However, sequencing studies of neuroblastoma tumors have revealed a low overall somatic mutation rate and few recurrently mutated genes (2-4). The young median age at diagnosis and standardized incidence ratio of siblings of children with neuroblastoma of approximately 9.7 (5) are consistent with an underlying genetic etiology.

The genetic basis of neuroblastoma predisposition has come into focus over the past decade. Familial neuroblastoma, which accounts for 1%-2% of cases, arises primarily from pathogenic germline variants in *ALK* (6), with rarer neurocristopathy syndrome cases explained by germline pathogenic variants in *PHOX2B* (7,8). However, the vast majority of neuroblastomas appear to arise sporadically, without a family history. Genome-wide association studies (GWAS) have identified common variants associated with sporadic neuroblastoma at more than a dozen loci. These genetic associations have implicated multiple candidate genes including *CASC15*, *NBAT1*, *BARD1*, *LMO1*, *DUSP12*, *DDX4*, *IL31RA*, *HSD17B12*, *HACE1*, *LIN28B*, *TP53*, *RSRC1*, *MLF1*, *CPZ*, *MMP20*, *KIF15*, and *NBPF23* (9-17). Several susceptibility genes identified by GWAS not only influence disease initiation but also drive tumor aggressiveness and/or maintenance of the malignant phenotype (11,13,15,18-21). A rare 16p11.2 microdeletion syndrome has also been associated with neuroblastoma (22). Finally, recent sequencing efforts have reported rare pathogenic germline variants in multiple cancer predisposition genes (2,23-33); however, the prevalence and clinical significance of these and other rare variants in neuroblastoma remain unclear and require evaluation in larger patient cohorts with detailed phenotypic data.

Here, we analyzed germline whole genome sequencing, whole exome sequencing, and targeted capture sequencing data from 786 children diagnosed with neuroblastoma and profiled through the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) initiative. Our aims were to 1) determine the prevalence, spectrum, and pathogenicity of rare germline variants in known cancer predisposition genes; 2) test for enrichment of rare variants in children with neuroblastoma compared with cancer-free control populations; and 3) evaluate clinical features and outcomes in neuroblastoma patients with and without germline pathogenic or likely pathogenic variants in cancer predisposition genes to identify translational opportunities.

Methods

Detailed methods are provided in Supplementary Methods (available online). Briefly, the study cohort consisted of 786 neuroblastoma patients accrued through the Children's Oncology Group (COG) ANBL00B1 biology study, unselected for family history (see Table 1; Supplementary Table 1, available online). Germline DNA and matched diagnostic tumor DNA and RNA were sequenced through the TARGET initiative. The original set of tumor-normal pairs were sequenced with Complete Genomics whole genome sequencing (n = 134) and/or Illumina whole exome sequencing (n = 222), as previously described (2,34). A total of 59 samples were sequenced by whole genome sequencing, and whole exome sequencing provided internal validation. We have previously reported a small number of germline variants based on the whole exome sequencing cohort (2); however, an in-depth study of pathogenic germline variation in these children was not performed at that time. Germline DNA from an independent neuroblastoma cohort (n = 489) was sequenced using Illumina custom capture panels, including a germline panel newly designed for this study (n = 166 genes; Supplementary Table 2, available online). Cancer-free control data were obtained from the Penn Medicine BioBank (PMBB; n = 6295) (35,36) and the Genome Aggregation Database (gnomAD v2.1) without cancer (n = 15 708). Ancestry for neuroblastoma and PMBB participants was inferred through principal component analysis using matched germline DNA array data. Germline variants were called using Genome Analysis Toolkit (GATK) best practices (whole exome sequencing and custom capture data) or the Complete Genomics pipeline (v2) with custom filtering (37) (whole genome sequencing data) then annotated with SnpEff (38) (v4.3t) and ANNOVAR (39). For all cohorts (neuroblastoma, PMBB, and gnomAD), rare germline variants (<0.1% across each population in public control databases) in 166 cancer predisposition genes were then assessed for pathogenicity with a clinically focused pipeline incorporating ClinVar (40) evidence and a modified implementation of InterVar (41), a tool that seeks to automate pathogenicity classification based on guidelines from the American College of Medical Genetics and Genomics and the Association of Molecular Pathology (42) (Supplementary Methods, Supplementary Figure 1, available online). A subset of germline variants were validated through Sanger sequencing. Patients harboring a germline pathogenic or likely pathogenic variant in a cancer predisposition gene were further assessed using matched tumor sequencing data when available. Fisher's exact test was used to compare the enrichment of pathogenic or likely pathogenic variants in neuroblastoma patients with 2 cancer-free control cohorts and across clinical and biological subsets of neuroblastoma. Pathogenic or likely pathogenic variant enrichment was also compared at gene

Table 1. Clinical and tumor biological characteristics for 786 neuroblastoma patients[a]

Characteristics	Neuroblastoma sequencing cohort No. (%)
Age	
Younger than 18 months	242 (30.8%)
18 months and older	544 (69.2%)
Sex	
Female	339 (43.1%)
Male	447 (56.9%)
COG risk	
Low	103 (13.2%)
Intermediate	119 (15.1%)
High	564 (71.8%)
INSS stage	
Stage 1	38 (4.8%)
Stage 2	60 (7.7%)
Stage 3	93 (11.8%)
Stage 4	546 (69.5%)
Stage 4S	49 (6.2%)
MYCN status	
Not amplified	552 (71.1%)
Amplified	224 (28.9%)
Not available	10[b]
Histology	
Favorable	211 (28.8%)
Unfavorable	522 (71.2%)
Not available	53[b]
Ploidy	
Hyperdiploid	473 (61.7%)
Diploid	293 (38.3%)
Not available	20[b]

[a] COG = Children's Oncology Group; INSS = International Neuroblastoma Staging System.
[b] Not included in % calculation

and pathway levels, using a Bonferroni correction for multiple testing. Kaplan–Meier analyses of event-free and overall survival were performed to compare outcomes of patients with and without germline pathogenic or likely pathogenic variants. A multivariate Cox proportional hazards regression model was used to assess if the presence of a cancer predisposition gene pathogenic or likely pathogenic variant was independently predictive of survival.

Results

Neuroblastoma patient characteristics

A total of 786 neuroblastoma patients were included in the study (Table 1; Supplementary Table 2, available online). Overall, 564 (71.8%) patients were classified as high-risk based on the COG risk stratification system, and 546 of these patients had stage IV disease according to the International Neuroblastoma Staging System (INSS) criteria. Cases profiled by whole genome sequencing or whole exome sequencing were intentionally enriched for high-risk disease, consistent with the overall goals of the TARGET initiative. In contrast, cases that underwent targeted capture (custom capture) sequencing were representative of the general neuroblastoma risk group profile. A total of 769 patients had available matched germline DNA array data and were evaluated for ancestry by principal component analysis. As expected, the majority (66.8%) of cases were inferred to be of European ancestry (Supplementary Figure 2, available online).

Frequency of pathogenic or likely pathogenic variants in known cancer predisposition genes

We observed 116 pathogenic or likely pathogenic variants involving 54 of the 166 cancer predisposition genes studied (Figure 1; Supplementary Table 3, available online). Of these variants, 73 were classified as pathogenic or likely pathogenic on the basis of ClinVar evidence, and 43 variants were assigned pathogenic or likely pathogenic on the basis of our revised InterVar assessment. Overall, pathogenic or likely pathogenic variants were detected in 109 of 786 (13.9%) neuroblastoma patients. Classic familial neuroblastoma germline variants were observed in 0.4% (3 of 786) of cases. These included 2 patients with *ALK* (p.R1275Q) activating variants and a single patient with a *PHOX2B* splice variant (NM_003924: exon3: c.430-2A>G). An additional *ALK* variant (p.I1250T) was predicted to be likely pathogenic; however, this variant was not found to be activating in a previous study (43). Six cases harbored more than 1 pathogenic or likely pathogenic variant in a known cancer predisposition gene (Supplementary Table 4, available online). A total of 27 genes harbored pathogenic or likely pathogenic variants in 2 or more cases, with variants in the *BARD1* gene being most frequent (8 of 786 cases or 1.0% overall; Supplementary Figure 3, Supplementary Table 3, available online). Select variants were validated by Sanger sequencing (Supplementary Table 5, available online). Notably, of the 9 neuroblastoma genes identified by GWAS and included in this study, only *BARD1* and *TP53* harbored rare pathogenic or likely pathogenic coding variants. This suggests that causal variants at other GWAS loci may be in the noncoding genome or tied to common variants not considered here.

Matched tumor DNA sequencing reveals pathogenic or likely pathogenic variants are retained and second hits are rare

We examined somatic alterations affecting genes with germline pathogenic or likely pathogenic variants using matched published neuroblastoma tumor whole genome sequencing and whole exome sequencing variant calls (34) and our analysis of available custom capture sequencing from TARGET. Nearly all (95%, 73 of 77) germline pathogenic or likely pathogenic variants were detected in matched tumor DNA, when available (Supplementary Table 3, available online). Pathogenic or likely pathogenic variants detected in tumor DNA had an average variant allele fraction of 0.46. In contrast, variants not detected in the matched tumor (n = 4) exhibited a lower variant allele fraction (range = 0.25-0.31), suggesting these variants may be mosaic. No second hit single nucleotide variations (SNVs) or indels were observed in the tumor DNA of patients harboring a germline pathogenic or likely pathogenic variant in a cancer predisposition gene. We detected 1 focal somatic deletion within *EZH2* observed in the tumor from patient PASEGA, who also harbored a pathogenic germline *EZH2* variant (p.T536fs). Phase could not be determined, but this somatic event was confirmed by Sanger sequencing of the tumor DNA (Supplementary Figure 4, available online).

Germline pathogenic or likely pathogenic variants in cancer predisposition genes are enriched in neuroblastoma patients

To assess whether children diagnosed with neuroblastoma harbor an excess of rare pathogenic germline variation in the 166 cancer predisposition genes considered in this study, we compared the pathogenic or likely pathogenic burden in neuroblastoma patients to 2 independent control cohorts (Figure 2, A, top panel). First, we applied our full analytic pipeline (alignment, variant calling, quality control, and pathogenicity assessment) to participants sequenced through the PMBB and without a history of cancer or benign tumors (n = 6295; Supplementary Figure 5, available online). Germline pathogenic or likely pathogenic variants were statistically significantly enriched in neuroblastoma patients compared with PMBB ($P_{PMBB} = 5.14 \times 10^{-5}$; odds ratio [OR] = 1.60, 95% confidence interval [CI] = 1.27 to 2.00). To assess reproducibility of this result, identical filtering and pathogenicity assessment was applied to rare variants in gnomAD excluding cancer samples (n = 15 708 individuals). This confirmed the excess burden of pathogenic or likely pathogenic variants in neuroblastoma ($P_{gnomAD} = 1.82 \times 10^{-3}$; OR = 1.41, 95% CI = 1.34 to 1.74).

Germline pathogenic or likely pathogenic variants in *BARD1* are enriched in neuroblastoma patients

Next, we performed gene-based rare variant burden testing comparing neuroblastoma patients with PMBB and gnomAD control cohorts (Supplementary Tables 6 and 7, available online). Five genes (*BARD1*, *EZH2*, *ALK*, *PTCH1*, and *MSH3*) exhibited statistically significant enrichment ($P < .05$), in both control cohort comparisons (Figure 2, B). Pathogenic variants in *BARD1* and *EZH2* were validated by Sanger sequencing in neuroblastoma patients when DNA was available (Supplementary Figures 3 and 4, available online). *ALK* and *BARD1* remained statistically significant after Bonferroni adjustment for multiple testing in at least 1 control comparison. *ALK* is the main major familial neuroblastoma predisposition gene (6). Only 1 likely pathogenic variant in *ALK* was detected in PMBB ($P_{PMBB} = 5.00 \times 10^{-3}$; OR = 24.09, 95% CI = 1.93 to 1255.78). This variant (p.A1168T) was classified likely pathogenic based on InterVar and was not reported in ClinVar. No pathogenic or likely pathogenic *ALK* variants were observed in gnomAD v2.1 whole genome controls ($P_{gnomAD} = 1.08 \times 10^{-4}$; OR = 140.3, 95% CI = 7.24 to 2719.0).

BARD1 is the only gene that passed a Bonferroni adjustment in both control comparisons (Figure 2, B). Common variation at the

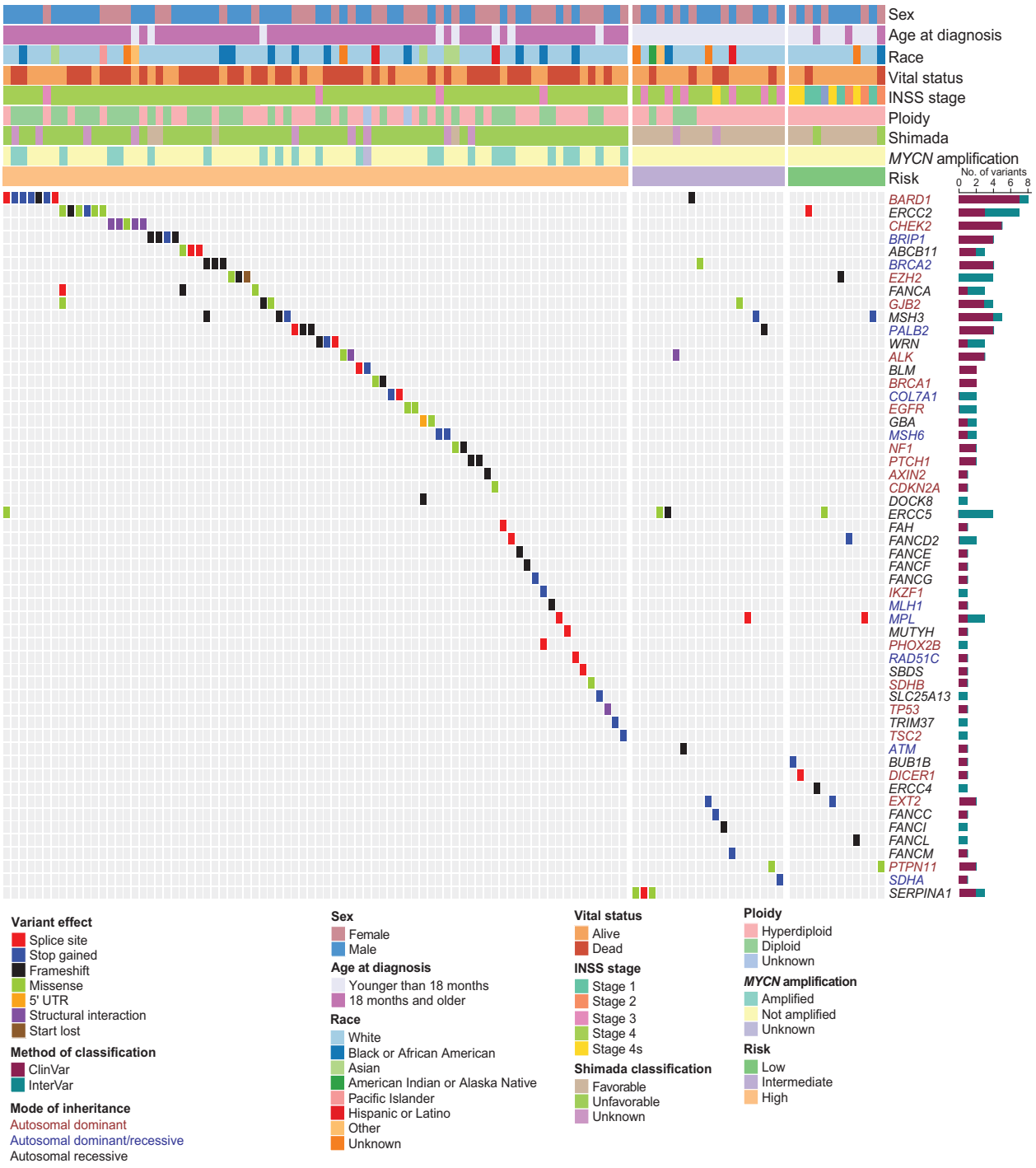

Figure 1. Germline pathogenic or likely pathogenic variants in cancer predisposition genes across 786 neuroblastoma patients. Oncoprint of known cancer predisposition genes harboring rare germline variants classified as pathogenic or likely pathogenic. Patients without pathogenic or likely pathogenic variants in these genes are not shown. Patients are ordered by Children's Oncology Group risk group and annotated with clinical and tumor biologic features. Genes are color-coded according to mode of inheritance, when known. Bar chart to the right indicates the number of variants detected for each gene and whether pathogenicity was determined based on ClinVar or our modified InterVar automated assessment. All variants were manually reviewed for quality and evidence of pathogenicity. INSS = International Neuroblastoma Staging System; UTR = Untranslated Region.

BARD1 locus is known to be associated with high-risk neuroblastoma from our prior GWAS (10). We and others have also reported germline *BARD1* rare variants in neuroblastoma patients (2,44,45). However, to date, the number of patients analyzed has been limited. Here, we observed rare pathogenic or likely pathogenic variants in *BARD1* in 8 of 786 (1.0%) neuroblastoma patients, all predicted to be loss-of-function (Figure 2, C; Supplementary Table 8, available online). Moreover, all but 1 variant was observed in the high-risk subset (Figure 2, D). Only 2 of 6295 (0.03%) control participants in PMBB harbored a pathogenic or likely pathogenic germline variant in *BARD1* ($P_{PMBB} = 8.18 \times 10^{-7}$; OR = 32.30, 95% CI = 6.44 to 310.35). Similarly, only 15 of 15 708 (0.09%) control participants in gnomAD harbored a pathogenic or likely pathogenic germline variant in *BARD1* ($P_{gnomAD} = 6.64 \times 10^{-6}$; OR = 10.75, 95% CI = 3.93 to 27.13). Enrichment of pathogenic or likely pathogenic variants in *BARD1* remained statistically significant when we

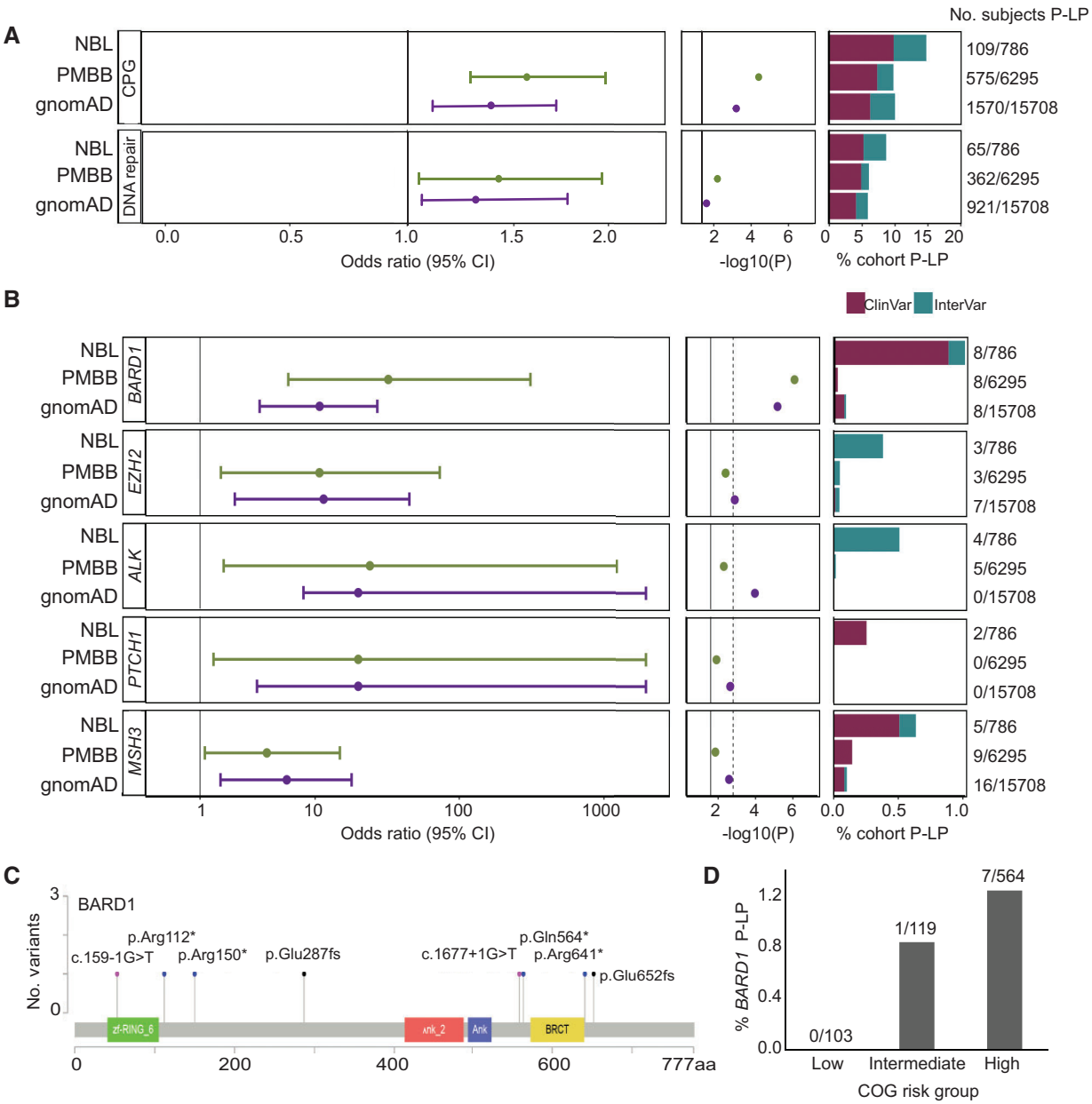

Figure 2. Neuroblastoma patients harbor an excess burden of rare pathogenic or likely pathogenic (P-LP) variants in cancer predisposition genes. **A**) Overall excess burden of P-LP variants (single nucleotide variations and indels) in neuroblastoma (NBL) vs Penn Medicine BioBank (PMBB) and the Genome Aggregation Database (gnomAD) v2.1 controls is shown for cancer predisposition genes and the subset of genes studied involved in DNA repair. **B**) Gene-based rare variant burden test results comparing the number of neuroblastoma subjects with P-LP variants to those detected in gnomAD v2.1 and PMBB. **C**) Lollipop figure depicting 8 germline P-LP variants in *BARD1*. **D**) Rare P-LP variants in *BARD1* are observed predominantly in patients diagnosed with high-risk neuroblastoma. CI = confidence interval; COG = Children's Oncology Group; CPG = cancer predisposition gene.

restricted the analysis to neuroblastoma patients and cancer-free control particpants of European ancestry, considering PMBB (P_{PMBB} = 2.70 x 10^{-5}; OR = 21.36, 95% CI = 4.55 to 131.80) and gnomAD (P_{gnomAD} = 1.55 x 10^{-4}; OR = 10.13, 95% CI = 3.75 to 28.74.86), suggesting this result is not likely due to population stratification.

Germline pathogenic or likely pathogenic variants in DNA repair genes are enriched in neuroblastoma patients

BARD1 is known to bind *BRCA1* and influence DNA repair (46). We hypothesized that neuroblastoma patients harbor an excess burden of pathogenic or likely pathogenic variants in DNA repair pathway genes overall. To explore this hypothesis, we interrogated genes in our 166-gene panel that intersected published DNA repair genes (47). A total of 48 DNA repair genes were assayed in the full study cohort and included in the analysis (Supplementary Table 2, available online). We observed 68 pathogenic or likely pathogenic variants in 27 distinct DNA repair genes, affecting 64 of 786 (8.1%) neuroblastoma patients (Figure 2, A, bottom panel). In contrast, only 362 of 6295 (5.8%) PMBB participants harbored a pathogenic or likely pathogenic variant (P_{PMBB} = 0.011; OR = 1.45, 95% CI = 1.08 to 1.92). Similarly, only 959 of 15 708 (6.1%) gnomAD participants harbored a pathogenic or likely pathogenic variant in a DNA repair gene (P_{gnomAD} = 0.028; OR = 1.36, 95% CI = 1.03 to 1.77).

Germline copy number variants disrupting *BARD1* are enriched in neuroblastoma

Given the enrichment of *BARD1* rare pathogenic or likely pathogenic SNVs and indels in this study and the previous association of *BARD1* common variants with neuroblastoma through GWAS, we next sought to determine if rare germline copy number variants at *BARD1* also associate with neuroblastoma. We analyzed copy number variants in germline DNA array data from 5585 neuroblastoma patients and 23 505 cancer-free control children genotyped in our neuroblastoma GWAS efforts (22). We detected 3 focal germline deletions fully or partially encompassing *BARD1* in neuroblastoma (0.05%; Figure 3, A-E; Supplementary Table 9, available online). No copy number variants affecting *BARD1* were observed in 23 505 chip-matched cancer-free GWAS participants, and no protein coding deletions affecting *BARD1* were observed in 10 847 individuals in the gnomAD v2.1 structural variant dataset (48) (Figure 3, A). The rare germline deletions at *BARD1* were statistically significantly associated with neuroblastoma ($P = 7.08 \times 10^{-3}$; OR = 29.47, 95% CI = 1.52 to 570.70; Figure 3, F) and were detected only in high-risk neuroblastoma patients (Figure 3, G). Collectively, these data

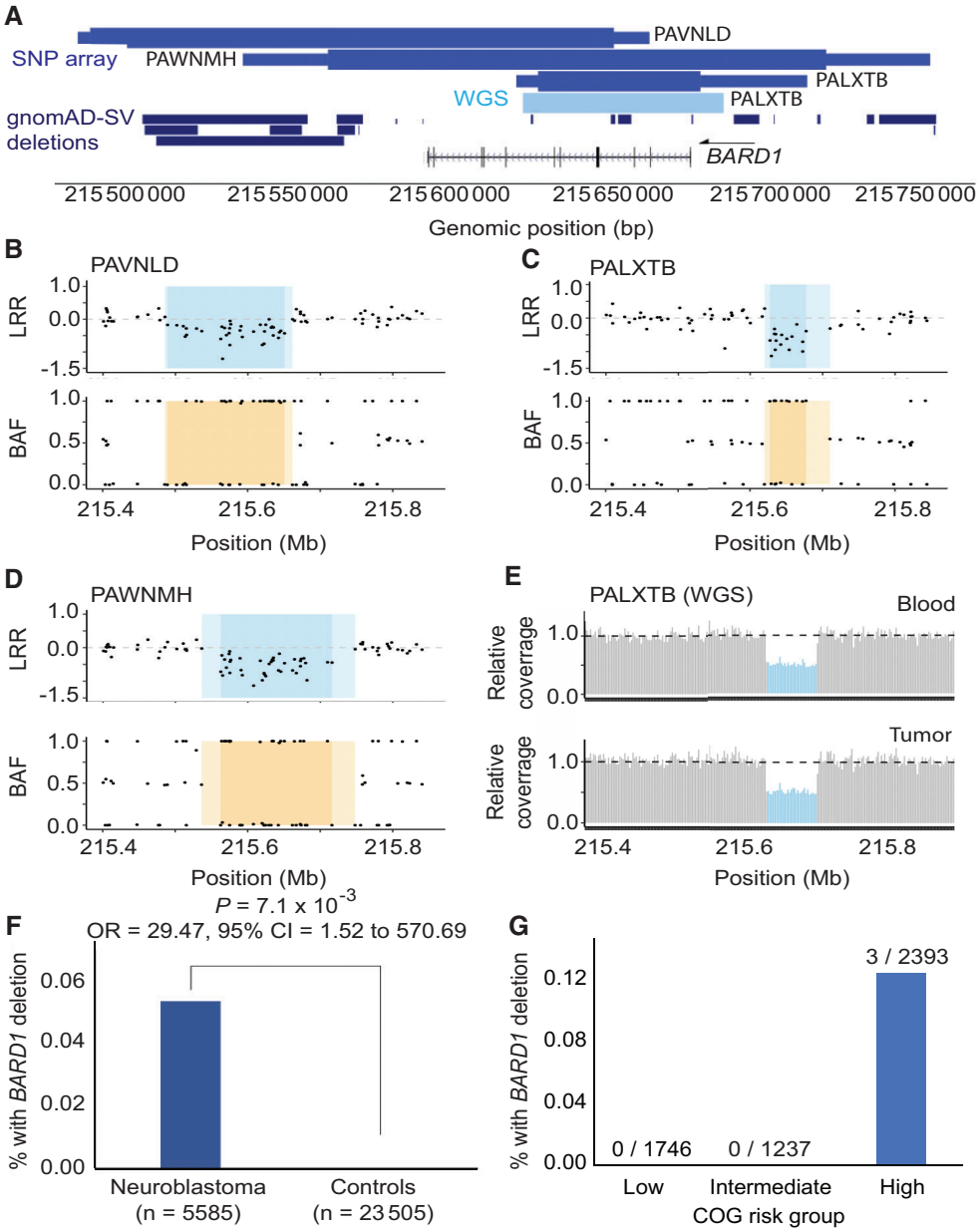

Figure 3. Rare germline copy number variants disrupting *BARD1* in neuroblastoma patients. **A**) *BARD1* deletions were identified in 3 of 5585 neuroblastoma patients through copy number analysis of a large germline single nucleotide polymorphism (SNP) array dataset (**medium blue, top track**). No deletions were observed in 23 505 array-matched cancer-free control participants. The **thick** and **thin bars** represent minimum and maximum deletion coordinates, respectively. One deletion was validated and fine-mapped by whole genome sequencing (WGS; **light blue, middle track**). No *BARD1* protein coding deletions were observed in 10 847 individuals in the gnomAD v2.1 structural variant dataset (**dark blue, bottom track**). **B-D**) The 3 array-based copy number variant calls are shown in log R ratio (LRR) and B allele frequency (BAF) plots. **Darker shading** indicates the minimum deleted region, whereas **lighter shading** indicates the maximum region. **E**) WGS validation for patient PALXTB is shown as relative sequencing coverage for matched blood and tumor samples. **F**) Rare *BARD1* deletion copy number variants are enriched in neuroblastoma compared to cancer-free participants. **G**) Deletions disrupting *BARD1* were observed exclusively in patients diagnosed with high-risk subset of neuroblastoma. gnomAD = Genome Aggregation Database.

suggest that *BARD1* alterations are an important genetic determinant of neuroblastoma, including common germline variants and rare germline SNVs, indels, and structural variants.

Neuroblastoma patients harboring germline pathogenic or likely pathogenic variants in cancer predisposition genes have worse overall survival

Finally, we investigated whether rare pathogenic or likely pathogenic variants (SNVs and indels) in cancer predisposition genes were associated with specific clinical and tumor biological characteristics and patient survival. A nominally statistically significant enrichment of pathogenic or likely pathogenic variants was observed in patients with tumors harboring loss of heterozygosity of chromosome 11q ($P = .012$); however, no association with age at diagnosis, stage, *MYCN* amplification status, COG risk group, or other characteristics was detected (Supplementary Table 10, available online). We repeated this analysis using the custom capture data only; however, the results were similar (Supplementary Table 11, available online). We next evaluated overall survival probability based on the presence or absence of germline pathogenic or likely pathogenic variants in cancer predisposition genes. We observed that patients with a germline pathogenic or likely pathogenic variant have worse overall survival compared with subjects without a germline pathogenic or likely pathogenic variant in a cancer predisposition gene (log-rank test $P = 8.6 \times 10^{-3}$; Figure 4, A). Furthermore, if restricted to only low- and intermediate-risk patients, overall survival remains worse for patients with a germline pathogenic or likely pathogenic variant (log-rank test: $P = 1.3 \times 10^{-4}$; Figure 4, B). A similar trend was observed when restricted to high-risk only, though this did not reach statistical significance (log-rank test $P = .1049$; Figure 4, C). Finally, a multivariate Cox proportional hazards regression model revealed the presence of a germline pathogenic or likely pathogenic variant was independently predictive of overall survival when considering age and diagnosis, INSS stage, *MYCN* amplification status, and COG risk group (Figure 4, D; $P = .017$; hazard ratio = 1.44, 95% CI = 1.07 to 1.96; Supplementary Table 12, available online). Taken together, these data demonstrate that the presence of a germline pathogenic or likely pathogenic variant in a cancer predisposition gene is associated with worse survival, independent of risk group.

Discussion

Neuroblastoma is a cancer of the developing sympathetic nervous system with an established genetic basis. Patients who present with a family history of the disease most commonly harbor rare pathogenic variants in *ALK* (6) or *PHOX2B* (7,8). In contrast, GWAS studies have identified common variation associated with sporadic neuroblastoma implicating more than a dozen susceptibility genes (49), including *BARD1* (10). Germline sequencing studies have reported rare pathogenic variation in cancer predisposition genes, including *APC*, *AXIN2*, *BARD1*, *BRCA1*, *BRCA2*, *CHEK2*, *LZTR1*, *PALB2*, *PINK1*, *SDHB*, *SMARCA4*, and *TP53* (50). Neuroblastoma has also been reported in several childhood-onset tumor-predisposition syndromes (50). However, a study of germline pathogenic variants in a clinically annotated cohort of patients large enough to investigate excess burden of pathogenic variation and clinical features associated with such variation has not been previously reported. Thus, in this study, we sought to define the prevalence, spectrum, and clinical significance of rare pathogenic germline variants in cancer predisposition genes in neuroblastoma.

To accomplish these goals, we analyzed germline DNA sequencing from 786 neuroblastoma patients with detailed clinical covariate and outcomes data. Using a conservative, clinically focused pipeline to classify pathogenicity of rare variants in cancer predisposition genes, we observed pathogenic or likely pathogenic germline variants in a substantial (13.9%) portion of neuroblastoma patients studied. This percentage is slightly higher but in-line with recent pan-childhood cancer germline studies (23,24,29,32,33,44). Two genes (*ALK* and *BARD1*) showed enrichment of pathogenic or likely pathogenic variants in neuroblastoma patients compared with independent cancer-free control cohorts after adjusting for multiple testing. Neuroblastoma patients carrying a germline pathogenic or likely pathogenic variant had worse overall survival compared with those without pathogenic or likely pathogenic variants, independent of age at diagnosis, INSS stage, *MYCN* amplification, and COG risk group.

The greatest number of pathogenic germline variants were observed in *BARD1*, *BRCA2*, *ERCC2*, *CHEK2*, and *MSH3*. Notably, *BARD1*, *BRCA2*, *CHEK2*, and *MSH3* variants were primarily classified as pathogenic or likely pathogenic based on ClinVar annotation, suggesting that these variants have previously been observed in patients in a clinical lab. All 5 genes are involved in DNA repair, and indeed we observed an overall enrichment of pathogenic or likely pathogenic variants in DNA repair genes considered here. Although this finding requires validation using a full repertoire of DNA repair genes, the result suggests that neuroblastoma is another cancer initiated by germline defects in DNA repair. The current study was large enough to demonstrate a statistically significant enrichment of rare *BARD1* pathogenic or likely pathogenic variants (SNVs and copy number variants) in neuroblastoma, adding to the common variants in *BARD1* identified by GWAS and previously implicated in disease pathogenesis. In addition to *BARD1*, we observed 1.7% (13 of 786) of children in our cohort with a pathogenic or likely pathogenic variant in *BRCA1*, *BRCA2*, or a mismatch repair gene, which is similar to that observed (approximately 1.2%) in a large (n = 3975) meta-analysis of childhood cancer studies (51). Notably, 2 of these patients harbored multiple pathogenic or likely pathogenic variants in these genes, including 1 patient with a pathogenic or likely pathogenic variant in both *BRCA1* and *BRCA2* and the other patient with pathogenic or likely pathogenic variants in *BRCA2* and *MSH3*.

In a parallel study, we used multiple lines of evidence to demonstrate the impact of *BARD1* germline pathogenic or likely pathogenic variants identified here on DNA repair processes in neuroblastoma (52). Briefly, a subset of the rare *BARD1* variants identified in the current study were introduced as monoallelic knock-ins in neuroblastoma cell models via CRISPR-Cas9 genome editing. These heterozygous variants induced *BARD1* haploinsufficiency, DNA repair deficiency, ineffective RAD51 foci formation at DNA double-strand break sites, and enhanced sensitivity to cisplatin and poly-adenosine diphosphate ribose polymerase inhibition. Taken together, these data further implicate *BARD1* and defective DNA repair as important driving factors in neuroblastoma tumorigenesis that may have important therapeutic implications.

Evidence for bi-allelic inactivation and/or loss of heterozygosity in neuroblastoma patients with pathogenic or likely pathogenic germline variants was observed in only 1 tumor and involved *EZH2*. There are several possible explanations for the

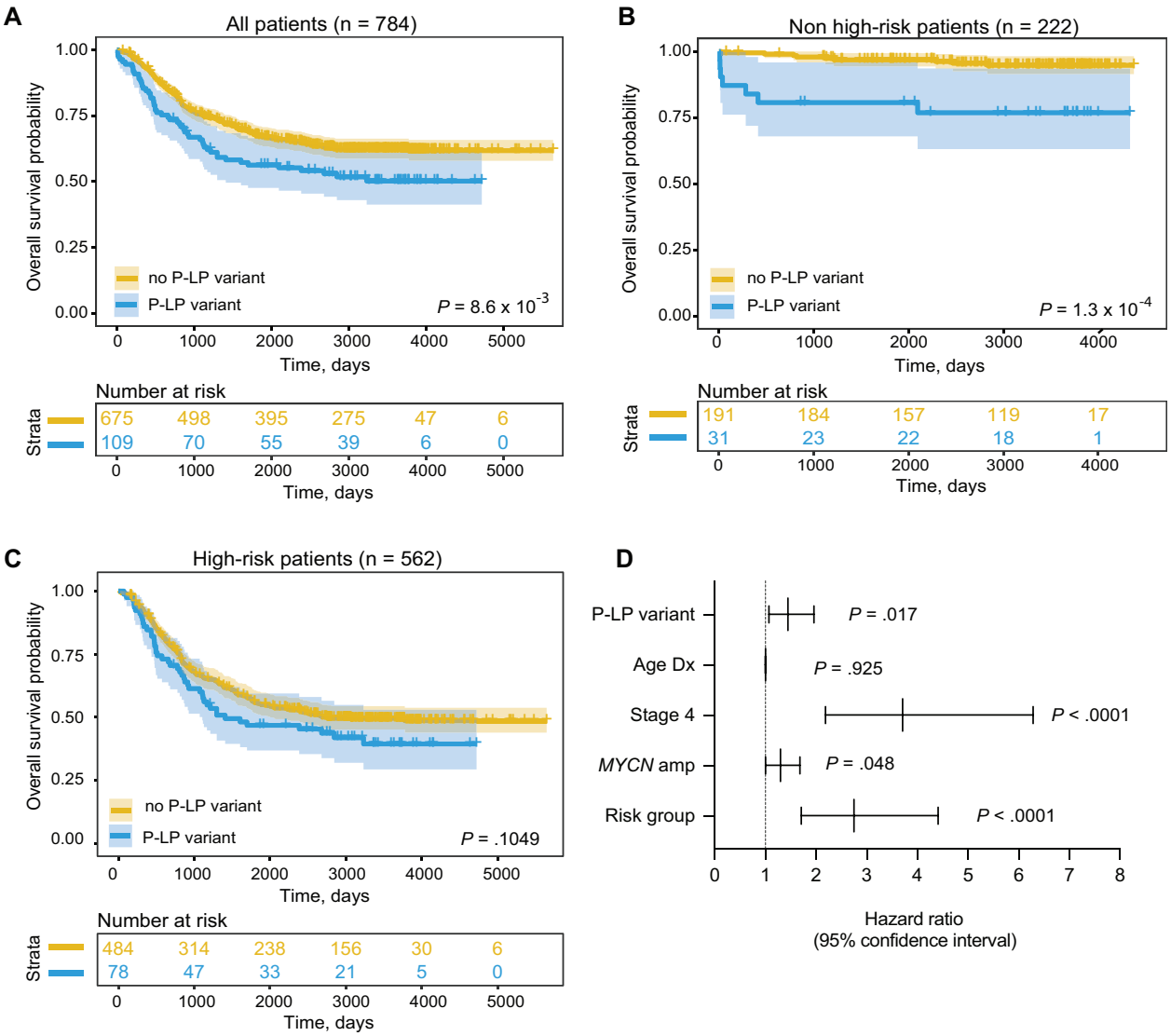

Figure 4. Neuroblastoma patients harboring germline pathogenic or likely pathogenic (P-LP) variants in cancer predisposition genes have worse overall survival. Kaplan–Meier plots of overall survival probability in neuroblastoma patients with and without P-LP variants cancer predisposition genes. **A**) All patients. **B**) Restricted to low- and intermediate-risk (non-high-risk) groups. **C**) Restricted to high-risk group. Statistical significance in panels **A-C** assessed by log-rank test ($P < .05$). **D**) Forest plot of hazard ratios from Cox proportional hazards model. amp = amplified; Dx = diagnosis.

low rate of bi-allelic inactivation in neuroblastoma. First, haploinsufficiency may be sufficient to tumorigenesis, as seen in our companion *BARD1* functional studies (52). Alternatively, other inactivation mechanisms may be present but not detected by our approach (eg, epigenetic and noncoding alterations). Finally, there have been a limited number of patients with germline pathogenic or likely pathogenic variants and matched tumor data evaluated to date. Functional studies, such as those presented for *BARD1* (52), and large cohort analyses incorporating the full spectrum of potential inactivation mechanisms are needed to resolve these important questions.

Universal germline and somatic genomic testing for adults with cancer has been advocated for using scientific and moral arguments (53). Similar arguments may also apply to patients diagnosed with neuroblastoma for multiple reasons: 1) from a prognostic standpoint, identification of a pathogenic or likely pathogenic variant predicts worse overall survival, independent of clinical risk stratification; 2) identification of pathogenic or likely pathogenic variants in some genes (eg, *BARD1*) may suggest eligibility for specific therapies, especially at time of relapse; 3) cascade testing of adult family members may guide gene-specific surveillance and therapies (eg, in *BARD1*, *BRCA1*, *BRCA2*, *CHEK2*, Lynch, *PALB2*, and *TP53*); 4) cascade testing of children for select genes (eg, *TP53*, *PTPN11*, and *DICER1*) may guide surveillance and therapy; and 5) identification of pathogenic variation in genes associated with specific syndromes (eg, *PTCH1* in Gorlin syndrome, *EZH2* in Weaver syndrome) may guide prognosis and clinical management. However, as the field is adopting paired germline-tumor DNA sequencing at the time of diagnosis for neuroblastoma and other pediatric cancer patients, there remains uncertainty on how to act on findings. This is particularly true for variants identified with low penetrance or lack of functional data or in clinical situations where access to high-quality genetic counseling is limited or there are challenges in performing effective surveillance (51). Genetic counseling should become an integral part of every patient's multidisciplinary cancer care planning, something that has already been implemented in many large academic centers. Moreover, centralization of

these data, through the Childhood Cancer Data Initiative or similar efforts, will facilitate longitudinal studies of patient survival and provide a resource for prioritizing functional and mechanistic studies to identify specific actionable insights to improve outcomes.

There are some limitations to this study. Patients studied were enrolled in the North American neuroblastoma biology study, ANBL00B1. These patients are predominantly of European ancestry and may not be representative of other geographic locations and ancestries. Second, we attempted to control for this in our burden testing; however, the use of different sequencing methods (whole genome sequencing, whole exome sequencing, custom capture) may affect variant detection at some genetic loci. Third, noncoding variants, epigenetic alterations, and an exhaustive set of structural variants were not analyzed here because of the different sequencing methods used. Finally, because of the lack of parental DNA, we cannot say whether the pathogenic or likely pathogenic variants identified in this study are inherited or acquired de novo. Patients in this study also did not include family history, multifocal tumor status, or secondary malignancy data. Additional large studies from diverse populations, including parental DNA and expanded annotations, are needed to replicate and extend our results. Recent sequencing studies, such as those supported by the Gabriella Miller Kids First research program, will be key in addressing many of these questions.

In conclusion, this study of 786 neuroblastoma patients found that 13.9% harbor rare germline pathogenic variants in 1 or more cancer predisposition genes. Rare pathogenic variants (SNVs and copy number variants) in *BARD1* and other DNA repair genes were statistically significantly enriched in neuroblastoma compared with cancer-free controls. The presence of 1 or more germline pathogenic or likely pathogenic variants in a cancer predisposition gene was independently associated with worse overall survival. These data may be used to inform decision making regarding genetic testing and potential therapeutic options for children diagnosed with neuroblastoma.

Data availability

All neuroblastoma sequencing data analyzed in this study are available through the database of Genotypes and Phenotypes (dbGaP; https:// www. ncbi. nlm. nih. gov/ gap/) under study-id phs000218 and accession number phs000467.

Author contributions

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Funding

This work was supported by a supplement to the Children's Oncology Group Chair's grant CA098543 and with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E to SJD and Complete Genomics. This work was also supported by National Institutes of Health grants R01CA204974 (SJD), R01CA237562 (SJD), R03CA230366 (SJD), R35CA220500 (M.M), K08CA230223 (KRB), K08CA215312 (KNM), a Howard Hughes Medical Institute Medical Fellows grant (MPR), Alex's Lemonade Stand Foundation (KRB), the EVAN Foundation (KRB), Burroughs Wellcome Fund—1017184 (KNM), Basser Center for BRCA (KNM), the Intramural Research Program of the Division of Cancer Epidemiology and Genetics and Center for Cancer Research of the National Cancer Institute, Bethesda, MD, and the Cancer Genome Research Laboratory, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, MD. KRB is a Damon Runyon Physician-Scientist supported (in part) by the Damon Runyon Cancer Research Foundation (PST-07-16).

Conflicts of interest

The authors have no disclosures.

Acknowledgements

This study involved collaboration with Penn Medicine BioBank and Regeneron Genetics Center. A complete list of individuals and their contributions is included in the Supplementary Information (available online). This work used resources of the NIH High Performance Computing Biowulf cluster and the Children's Hospital of Philadelphia High Performance Compute cluster. The funders had no role in the design of the study; the collection, analysis, or interpretation of the data; or the writing of the manuscript and decision to submit it for publication.

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