Replication of neuroblastoma SNP association at the *BARD1* locus in African-Americans

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Abstract

Background—Neuroblastoma (NBL) is an often-fatal pediatric cancer more frequent in European-American than African-American children. African-American children, however, are at higher risk for the more severe form of NBL, and have worse overall survival than European-American children. Genome wide association studies (GWAS) have identified several SNPs associated to NBL in children of European descent. Knowledge of their association to NBL in African-American children is still lacking.

Methods—We genotyped and imputed SNPs located in three gene regions reported to be associated to NBL in children of European descent, and tested them for association in 390 African-American NBL patients compared to 2500 healthy, ethnically matched controls.

Results—SNPs in the *BARD1* gene region show a similar pattern of association to NBL in African-American and European-American children. The more restricted extent of linkage disequilibrium in the African-American population suggests a smaller candidate region for the putative causal variants than previously reported. Limited association was observed at the other two gene regions tested, including *LMO1* in 11p15 and *FLJ22536* in 6p22.

Conclusions—Common *BARD1* SNPs affect risk of NBL in African-Americans. The role of other SNPs associated to NBL in children of European descent could not be confirmed, possibly due to different patterns of linkage disequilibrium or limited statistical power to detect association to variants with small effect on disease risk. Extension of GWAS to populations of African descent is important to confirm their results and validity beyond the European populations, and can help to refine the location of the putative causal variants.

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Keywords

Neuroblastoma; *BARD1*; African-Americans; SNPs; genetic association

Introduction

Neuroblastoma (NBL) is an important pediatric cancer with an age-adjusted incidence rate in Americans of European descent of approximately 11.5 per million (1). NBL is reported to be rarer among African-Americans (AA) children, with an age-adjusted incidence of 8.5 per million (1). Limited data exist on incidence of NBL in Africa, but reports suggest it is similar to that of AAs or lower (1). Odds-ratio of NBL by parental ethnic origin relative to European-American (EA) children was only 0.74 (95% CI 0.56–0.96) for children with both AA parents (2). However, a recent study has shown that AA children in the US are more likely to have the high-risk form of the disease compared to EA children (57% vs. 44%), and have associated lower overall survival probability (67% vs. 75%) (3). According to the Surveillance, Epidemiology, and End Results database (4), for ages less than 1 year the age-adjusted incidence is 58 per million in EAs (95% confidence interval (CI) 52–64) versus only 30 per million in AAs (95% CI 21–42); however incidence rates in the two ethnic groups are more similar for older ages (20 vs. 21 for ages 1–4; 4 vs. 3 for ages 5–9; 1 vs. 2 for ages 10–14). Older age at diagnosis is an adverse prognostic factor in NBL (5).

Genetic risk factors may contribute to disparities in cancer prevalence and outcome. In recent years, findings from case-control genome wide association studies (GWAS) (6–10) and family-based linkage analysis (11) have improved our understanding of genetic susceptibility to NBL. Individuals of European descent constitute the majority of NBL patients in the United States, and so far the GWAS of NBL have been limited to this ethnic group. Here, we have obtained genome-wide SNP genotyping data on a cohort of AA NBL patients collected by the Children's Oncology Group, and have used this information in a case-control study to evaluate whether the same genes and SNPs identified in the EA studies affect risk of NBL in AA children. Our results show that of all the risk variants identified so far, only SNPs of *BARD1* unequivocally have an effect on NBL risk in AA children. Whether this is due to difference in genetic susceptibility or limited power to detect small genetic effects remains to be determined.

Materials and Methods

Patients and controls

DNA samples and clinical information were available for 390 AA NBL patients from the Children's Oncology Group, with clinical and biological annotation as reported previously (6–10). For this study, subjects eligible for inclusion were AAs based on self-reported ethnicity. A total of 2500 control samples were selected based on self-reported AA ethnicity from the Center for Applied Genomics (CAG) at the Children's Hospital of Philadelphia.

Genotyping and quality control filters

Genome-wide SNP genotype data from 390 NBL patients and 2500 disease-free control subjects were obtained at the CAG using the Illumina HumanHap 550 (243 cases, 1875 controls) and 610 Quad (147 cases, 625 controls) SNP chips as previously described (9). Only SNPs that were included in both chips were evaluated further.

SNPs were excluded from analysis if they showed deviation from Hardy–Weinberg equilibrium with a p-value less than 10^{-4} in controls or 10^{-7} in cases, a genotype yield of

less than 95%, a minor allele frequency (MAF) of less than 1%, and difference in missing rates between cases and controls with a p-value less than 10^{-4}. This filtering resulted in a total of 504,535 autosomal SNPs available for the subsequent analyses. Among these, we selected all the SNPs included in three genes showing association to NBL in previous GWAS of EAs, namely *FLJ22536*, *BARD1*, and *LMO1* (6,7,9). We also included all the extra-genic SNPs located in the regions extending 10 Kb on either side of each candidate gene, or as far as the most distant SNP having r^2 0.5 in the HapMap CEU group with the NBL associated SNPs, whichever was larger. This brought the total number of genotyped SNPs to be tested for association to 201 (Supplemental Table 1). Coverage of common variants in the candidate regions provided by these SNPs was estimated using HapMap Phase II data in the CEU and YRI populations using the Tagger option of Haploview (12) with pairwise tagging at $r^2=0.8$.

We did not include in our analysis SNPs from the regions that were identified as NBL associated in the low-risk patients only (including the genes *DUSP12, DDX4, IL31RA*, and *HSD17B12*) (10) because of the small size of this group in our sample (97 patients).

Prior to statistical analysis, individual data were filtered on the basis of standard quality control measures, including call rates, discrepancy between reported sex and X chromosome marker heterozygosity, and cryptic relatedness. A total of 34 samples (25 case subjects and 9 control subjects) had genotype yields of less than 95% and were removed, leaving 365 cases and 2491 controls available for analysis. Power to detect association in this sample given varying risk allele frequency (0.1–0.9) and genetic risk effect (1.2–1.6)was estimated using Quanto (13).

Analysis of stratification and admixture

AAs are a recently admixed population with two major founder groups. Admixture may introduce a bias in association tests if ancestry proportions are different between cases and controls. Ancestry proportions in our cases and controls were estimated using the software ADMIXTURE (14) and assuming 2 founder populations. We also evaluated stratification in our case-control sample by multidimensional scaling (MDS) using the procedure implemented in PLINK (15) and a subset of independent SNPs ($r^2<0.01$). Data from our cases and controls were compared to those obtained from the HapMap in the 4 major populations (CEU, CHB, JPT, YRI).

Association analysis

To account for possible difference in substructure between cases and controls due to varying levels of admixture, we tested SNPs for association with NBL by logistic regression using the proportion of African ancestry estimated by ADMIXTURE as covariate. We also tested for association using the stratified Cochran-Mantel-Haenszel (CMH) test implemented in PLINK based on the clusters identified by the MDS analysis (15). Results of the association tests with the two methods were almost identical and only results of logistic regression are presented in details (CMH results available in Supplemental Table 1).

To correct for the different number of SNPs tested in each candidate region, while taking into consideration the correlation between SNPs in any given region due to linkage disequilibrium (LD), we estimated empirical p-values by permutation of case-control status separately in each gene, using the EMP2 statistic from the mperm procedure implemented in PLINK (15). P-values reported in our tables are asymptotic p-values from the corresponding tests and empirical p-values from 10,000 permutations. Since all three genes have been previously reported to be associated to NBL in multiple independent datasets (6,7,9), we did not correct for the number of genes tested.

Regional association and LD plots were generated using SNAP (16).

Imputation

Imputation was performed with IMPUTE2 (17) using the complete 1000 Genomes Project Phase 1 interim data as reference (18). We followed the practice recommended by the developers of IMPUTE2 to use a cosmopolitan reference population rather than arbitrarily select ancestral populations for our individuals (19). All SNPs contained in the reference data and included in the three gene regions defined above were imputed. Following imputation, SNPs with MAF <1% or IMPUTE2-info quality score <0.8 were removed. To correctly account for uncertainty in the data resulting from the imputation process, the remaining SNPs were tested for association with NBL using the frequentist association test under the additive model implemented in SNPTEST (20), including the estimated proportion of African admixture as covariate. All genotyped SNPs were also tested with the same test to compare results with the imputed SNPs.

Results

Power and SNP tagging

Power to detect association in our study was estimated to be >80% for intermediate risk allele frequencies (0.2–0.7) and odds-ratios over 1.4 (Supplemental Figure 1a).

Percentage of HapMap release 24 SNP coverage for the individual gene regions provided by the SNPs included in our analysis varied from 49% for *FLJ22536* to 64% for *BARD1* in the YRI population, and from 70% for *LMO1* to 77% for *FLJ22536* and *BARD1* in the CEU population (Supplemental Figure 1b).

Stratification and admixture

MDS analysis of cases and controls together with the 4 major HapMap populations showed that our samples cluster along a continuum between the CEU and the YRI populations as expected (Supplemental Figure 2a, b). Genome-wide estimates of African ancestry assuming two founder populations had a mean of 0.76%±0.23 in cases and 0.76%±0.19 in controls. There were some slight differences between the two groups with more cases than controls at the extremes of the distribution (0–10% and 80%–90% African ancestry) (Supplemental Figure 2c).

Association analysis of genotyped SNPs

Based on the genome-wide data, we estimated a genomic inflation factor (GIF) of the logistic regression test of 1.02. In contrast, the GIF of the 1 degree-of-freedom allelic test was 1.19. These data suggest that the procedures implemented to control population stratification were effective in reducing any potential inflation in type 1 error.

Results for the most significant SNPs reported in previous GWAS of EA patients (or index SNPs) are reported in Table 1. In the AA case series, two SNPs at the *BARD1* locus, rs7587476 and rs6435862, showed significant p-values even after multiple test correction (permutation p=3×10^{-4} and 5×10^{-4} respectively), and the other 3 *BARD1* index SNPs reached nominal significance. For all 5 *BARD1* index SNPs, the direction of the association was the same as the one observed in the EA patients, with similar odds-ratios in the range of 1.3 to 1.5 (8). None of the index SNPs in the other genes reached even nominal levels of significance (all nominal p-values > 0.05).

Because our previous studies in EAs had found that association signals were stronger when analysis was restricted to high-risk patients in spite of the smaller sample size (6,7,9), we

repeated the association analysis in this subgroup (180 cases) against all controls. Of the two significant index SNPs of *BARD1* from the analysis of all patients, one became more significant (rs7587476, permutation $p<10^{-4}$), and the other less (rs6435862, permutation $p=10^{-3}$). All other *BARD1* index SNPs were not significant after multiple test correction (permutation p>0.05) (Supplemental Table 1). One index SNP in *LMO1* reached nominal significance in the high-risk group (rs294938, p=0.04). However the direction of the association was the opposite of that observed in the EAs, where the G allele showed association to NBL (9), rather than the A allele as in the AAs. All the other index SNPs were still not significant (all nominal p-values > 0.05) (Supplemental Table 1).

We then asked whether other SNPs in the candidate regions, different from those reported as most significant in the EA patients, reached statistical significance in the AA patients. One *BARD1* SNP (in addition to those already reported in Table 1) showed significant association (rs16852804, permutation p=0.02). When we looked at the high-risk subgroup separately, one additional *BARD1* SNPs (rs7599060, permutation p=0.02) and one SNP in *LMO1* (rs4237769, permutation p=0.001) reached statistical significance. No other SNP showed statistical significance after multiple test correction (Supplemental Table 1). No additional signals of association were detected when analysis was further extended to include all SNPs with $r^2>0.1$ with the index SNPs (all p>0.05).

Overall, three SNPs of *BARD1* reached statistical significance after correction for the number of SNPs tested, and 10 more had nominal p-values less than 0.05 (Supplemental Table 1 and Supplemental Figure 3a). To test whether these may represent multiple independent signals of association, we repeated the logistic regression test conditional on the most significant SNP, rs7587476. None of the SNPs was significant after multiple test correction (permutation p-values>0.05). The nominal p-value for rs6435862 went from 0.00002 to 0.04, and for rs16852804 from 0.0007 to 0.005. All other nominal p-values were greater than 0.05 (Supplemental Figure 3b).

To examine the extent of the associated region, we plotted the r^2 values relative to rs7587476 for all the SNPs in a 100Kb region around it against their genomic location. We chose rs7587476 as the reference SNP because not only is the most strongly NBL associated SNP in this study, but is also the most significant *BARD1* SNP in our most recent analysis of EAs (9). Using data from the 1000 Genomes Project Pilot 1 in the CEU and YRI populations and the web-based software SNAP (15), we determined the size of the region which includes SNPs with $r^2 >0.5$ with rs7587476 (Supplemental Figure 4). In the YRI population, this region extends from 215,348,641 to 215,367,140 bp and comprises introns 2–4 and exons 3–4 of BARD1. In the CEU population, this region extends from 215,344,039 to 215,457,501 bp, and thus for an additional 4.6 Kb proximally and 90.4 Kb distally.

Association analysis of imputed SNPs

Following imputation using reference data available through the 1000 Genomes Project Phase 1, the number of imputed SNPs that passed quality control filters (MAF 1% and IMPUTE2-info score 0.8) was 948 for the *BARD1* region, 1946 for the *FLJ22536* region, and 215 for the *LMO1* region (Supplemental Table 2).

One *BARD1* imputed SNP, rs35933323, located in intron 1, had a slightly smaller p-value than the most significant genotyped SNP, rs7587476 ($p=1\times10^{-6}$ and $p=2\times10^{-6}$ respectively), and eight additional imputed SNPs had smaller p-values than rs6435862, the second most significant genotyped SNPs ($p<1.6\times10^{-5}$) (Supplemental Figure 5a). All these SNPs are intragenic to *BARD1* and have r^2 values with rs7587476 ranging from 0.51 to 1 in the CEU samples and from 0.24 to 1 in the YRI samples.

In the *FLJ22536* gene region, the most significant imputed SNP was rs9460668, with a p-value of 0.00015. An additional 82 imputed SNPs had p-values smaller than that of the most significant genotyped SNP, rs9466182, which had a p-value of 0.013, and of these, 50 had a p-value <0.01 (Supplemental Figure 5b). None of the imputed SNPs in the *LMO1* region had smaller p-values than rs4237769, the most significant genotyped SNP (p-value=0.0054) (Supplemental Figure 5c).

Discussion

SNPs in the *BARD1* gene region associated to NBL in EA patients show similar, strongly significant association in AA patients. In particular among the genotyped SNPs most of the association seems to be explained by rs7587476, located in intron 3 of *BARD1*, with some residual association signal detected by SNPs located in the first intron (Supplemental Figure 3). Analysis of imputed SNPs identified additional SNPs strongly associated to NBL in the same intragenic region, but limited association outside the gene's boundary (Supplemental Figure 5a). In contrast, the association in EAs extends further to the extra-genic region 5' to *BARD1* (9), possibly due to the more extensive LD around the associated SNPs (Supplemental Figure 4). This information may be helpful in locating the causal *BARD1* variants.

Besides the *BARD1* SNPs, the only other genotyped SNP that remained significant after correction for number of SNPs tested in a given gene was rs4237769 in *LMO1* (permutation p=0.001), when analysis was restricted to patients from the high-risk group. Five other SNPs in or around *LMO1* had nominal p-values <0.05 in the high-risk group, including rs204938, previously reported associated to NBL (9). The second most significant *LMO1* SNP in the high-risk group in this study, rs3794012 (nominal p=0.005) had a p-value of 3×10^{-5} in Wang et al. (9). However, the rare G allele of rs3794012 is associated to NBL in the AA patients, rather than the common A allele as observed in the EA cases.

Interestingly we did not detect a clear signal of association with SNPs of the *FLJ22536* gene region, which show the most significant association to NBL in European-Americans (6,9). The most significant *FLJ22536* genotyped SNPs in our study were rs9466182 with a nominal p-value of 0.01 in the whole group of cases, and rs1207774 with a nominal p-value of 0.005 in the high-risk group. However these are hardly significant when considering the large number of SNPs tested in this gene (136 genotyped SNPs). The different structure and lower LD in the AA population may have prevented us to detect association in *FLJ22536*, as well as this study being relatively underpowered for this rare condition. Based on HapMap CEU and YRI data, among the regions studied, coverage of *FLJ22536* variation in Africans provided by the genotyped SNPs is the lowest (49%) and the most divergent from that of EAs (77%) (Supplemental Figure 1b). Analysis of over 1,900 imputed SNPs detected a more significant signal of association with rs9460668, with a nominal p-value of 0.00015 (Supplemental Figure 5b). Although there are no LD data available for rs9460668, this SNP is located approximately 400 kb away from the *FLJ22536* SNPs associated to NBL in EAs, and is unlikely to be in LD with them. Additional analyses with direct genotyping in this and other populations will be necessary to confirm whether this finding represents a real association or a false positive due to the large number of SNPs tested.

This study demonstrates the difficulty in replicating association in AAs even for SNPs that are confirmed and show strong significant association in multiple European or EA populations. Similar conclusions were reported for other well documented SNP associations following GWAS in breast and prostate cancer (21,22). Several factors may contribute to this discrepancy, including variation in LD patterns and allele frequencies among ethnic groups, allele and genetic heterogeneity, and low power to detect association to variants with

small effect. A more detailed analysis genotyping additional SNPs not included in the standard GWAS chips and a larger sample size may be necessary to be able to conclude on the role of these genes on susceptibility to NBL in AAs. A larger sample size would also allow the opportunity to evaluate association separately in subgroups stratified by the admixture proportions, such as those with a preponderance of European or African ancestry.

Nonetheless, attempting replication of known association findings in populations of African descent is important not only to establish their validity in these populations, but also as it can reduce the size of the candidate regions thanks to the more limited LD structure in these populations (23), as shown here in the case of *BARD1*. Association in the EA group extended to SNPs located in the extra-genic region 5' to *BARD1* as far as 75 Kb from its initiation codon. Based on our study, a much smaller candidate region for location of the putative causal variants can be identified limited to the intragenic region between introns 1 and 4 (Supplemental Figure 3). While our sample size may be still too small and the gene coverage too low to detect new significant associations which may be specifically relevant to AA patients, our results for *BARD1* clearly indicate the value to extend gene mapping studies to well characterized samples from this ethnic group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Results of logistic regression test of association in African-American NBL patients for most significant SNPs in European-American GWAS

Gene	Chr.	SNP (bp)	Alleles (m/M)	MAF cases	MAF controls	p-value	Emp-p	OR	95% CI
FLJ22536	6	rs4712653 (22233943)	T/C	0.19	0.19	0.56	1.00	0.94	0.77–1.15
		rs9295536 (22239908)	C/A	0.19	0.19	0.30	1.00	0.90	0.73–1.10
		rs6939340 (22247983)	A/G	0.17	0.18	0.77	1.00	0.97	0.79–1.19
BARD1 *	2	rs3768716 (215344039)	G/A	0.10	0.07	0.007	0.14	1.45	1.11–1.90
		rs17487792 (215351745)	T/C	0.10	0.06	0.009	0.19	1.45	1.10–1.93
		rs7587476 (215362132)	T/C	0.42	0.34	2.4×10^{-6}	0.0003	1.47	1.25–1.72
		rs6712055 (215375149)	C/T	0.22	0.17	0.006	0.14	1.31	1.08–1.59
		rs6435862 (215380791)	G/T	0.34	0.26	1.8×10^{-5}	0.0005	1.44	1.22–1.70
LMO1	11	rs4758051 (8195215)	A/G	0.20	0.18	0.77	1.00	1.03	0.84–1.27
		rs10840002 (8199602)	G/A	0.27	0.25	0.79	1.00	1.03	0.85–1.23
		rs110419 (8209429)	G/A	0.22	0.23	0.18	0.99	0.88	0.72–1.07
		rs204938 (8234773)	T/C	0.34	0.31	0.27	1.00	1.10	0.93–1.30

m/M = minor allele/major allele

MAF = minor allele frequency

Emp-p = empirical p-value from permutation test

OR = odds-ratio for the minor allele

CI = confidence interval

*
SNP rs6715570 reported by Capasso et al. (6) was removed from our analysis for missing rate greater than 5% in cases.