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Genetic susceptibility to neuroblastoma

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Abstract

Until recently, the genetic basis of neuroblastoma, a heterogeneous neoplasm arising from the developing sympathetic nervous system, remained undefined. The discovery of gain-of-function mutations in the *ALK* receptor tyrosine kinase gene as the major cause of familial neuroblastoma led to the discovery of identical somatic mutations and rapid advancement of *ALK* as a tractable therapeutic target. Inactivating mutations in a master regulator of neural crest development, *PHOX2B*, have also been identified in a subset of familial neuroblastomas. Other high penetrance susceptibility alleles likely exist, but together these heritable mutations account for less than 10% of neuroblastoma cases. A genome-wide association study of a large neuroblastoma cohort identified common and rare polymorphisms highly associated with the disease. Ongoing resequencing efforts aim to further define the genetic landscape of neuroblastoma.

Keywords

Genome-wide association study; Cancer; Pediatrics; Hereditary

Introduction

Neuroblastoma is the most common solid extracranial malignancy of childhood, accounting for about 7% of all cancers in children under the age of 15.[1] It is the most common cancer in the first year of life, with a median age of diagnosis of 17 months.[1,2] It is a cancer of the developing sympathetic nervous system, arising in the adrenal medulla or paraspinal ganglia.[3] Approximately 65% of these tumors present in the abdomen, along with the neck, pelvis and chest.[4] Clinical course can vary widely, with infants often having spontaneous regression of the tumor without chemotherapy,[5–8] while older children

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Conflict of Interest

The authors declare that there are no actual or perceived conflicts of interest.

generally have a poor prognosis despite highly intensive chemotherapy, radiation therapy, and immunotherapy.[4] Demonstrating the phenotypic heterogeneity of neuroblastoma, low-risk patients have a greater than 95% survival probability whereas high-risk patients have a 40–50% probability of long-term survival. [9,10] It has been known for some time that *MYCN* amplification in tumors portends a poor prognosis,[11–13] and thus is used as a biomarker for treatment stratification. Recently, there has been significant effort made to better classify subgroups of patients based on age, and tumor spread, genomics and differentiation.[14–18] The International Neuroblastoma Risk Group (INRG) classification has led to 16 statistically distinct risk groups based on clinical and molecular features which has made prognosis more accurate for patients and helps guide physicians on treatment regimens.[15]

Significant progress has been made recently in the understanding of the heritability of neuroblastoma through linkage scans of families with the disease and genome-wide association studies (GWAS) of sporadic cases (Table 1). The primary advantages of GWAS over previous methods are that no assumptions about candidate genes are necessary, variations can be localized precisely, and no testing in families or family members is required.[19] From a clinical standpoint, it is clear that improvement must continue to be made in defining novel therapeutic approaches to neuroblastoma as it continues to account for 12% of childhood cancer mortality,[10] with advancement especially crucial in high-risk patients.[20] One starting point to develop optimal treatments is to understand the underlying genetic alterations that initiate tumorigenesis. We review here the current understanding of the genetic susceptibility of neuroblastoma.

Familial Neuroblastoma

About 1–2% of neuroblastoma is inherited in an autosomal dominant fashion within families.[21–24] As with many cancer predisposition syndromes, patients often have multiple primary tumor sites and an earlier age of onset. The disease is typically highly penetrant, but there is variability and unaffected obligate carriers are often observed. [21,22,25] Neuroblastoma families often show significant clinical variability in severity of disease, with low- and high-risk cases observed in the same pedigrees.[26–30] While rare, these families provide a unique opportunity to learn about genetic drivers of neuroblastoma.

The first gene found to predispose to neuroblastoma was identified in families affected with neuroblastoma along with Hirschsprung disease and/or congenital central hypoventilation syndrome (also known as “Ondine’s Curse”). These disorders of neural crest-derived cells are known as neurocristopathies and are occasionally seen coincident with neuroblastoma. [31–35] Amiel and colleagues identified loss of function mutations in the paired-like homeobox 2B (*PHOX2B*) gene in the majority of patients with congenital central hypoventilation syndrome after sequencing this candidate gene.[36,37] This gene was of interest because the *PHOX2B* transcription factor is essential during development of the autonomic nervous system. Germline mutations in *PHOX2B* were subsequently found in a small proportion (~10%) of pedigrees with familial neuroblastoma, making this the first *bone fide* neuroblastoma predisposition gene.[38,39] As expected, the families with *PHOX2B* mutations also had variable penetrance of each of the component

neurocristopathies, with non-polyalanine repeat expansion mutations (NPARM) typically lead to the most severe phenotype. [40,41]

In order to identify additional hereditary predisposition genes in the familial neuroblastoma cases, a genome-wide linkage scan at 6,000 single nucleotide polymorphisms (SNPs) was undertaken in 20 neuroblastoma families.[25] A linkage signal was found and narrowed down to chromosome bands 2p23–p24, which contained 104 genes including *MYCN*. This known neuroblastoma oncogene was resequenced in all probands, but no mutation was found. The anaplastic lymphoma kinase (*ALK*) is also in this region and had been previously identified as a potential oncogene in this malignancy[42,43] as well as in other cancers through active translocations and point mutations.[44–51] When *ALK* was resequenced, three distinct mutations were found in this gene in eight discrete families.[25] Subsequent studies have confirmed that about 80% of families with neuroblastoma harbor mutations in *ALK*. Mutations in *ALK* were also found to be somatically acquired in about 10% of all cases of neuroblastoma.[25,52–54] *ALK* is a receptor tyrosine kinase, and all of these were activating mutations in the tyrosine kinase domain that caused constitutive phosphorylation and were predicted to be oncogenic drivers.[25] While Knudson and Strong's prediction of a "two-hit" model has held true for most hereditary cancers,[21] these susceptibility genes are usually tumor suppressor genes. In contrast, *ALK* was the first oncogene mutation shown to cause a familial pediatric cancer. The Mosse lab has subsequently biochemically characterized each of the germline and somatic mutations, and there is a correlation between penetrance and mutation type. [55,56] For example, the R1275Q mutation leads to near complete penetrance in families and was shown to be one of the most activating mutations tolerated in the germline, whereas the G1128A is more weakly activating and is correlated with an approximate 25% likelihood of developing neuroblastoma. Interestingly, the two most highly activating hotspot mutations acquired somatically (F1174* and F1245*) were each observed in the germline once, but in the setting of neuroblastoma with severe neurocognitive defects and brain stem abnormalities, further emphasizing the genotype-phenotype relationship as well as the critical role plays in normal neurodevelopment.[57] Genetic testing for both *ALK* and *PHOX2B* are currently available for identifying genetic susceptibility and informing decisions about screening other family members (<http://www.ncbi.nlm.nih.gov/sites/GeneTests/>).

ALK was quickly identified as a potential pharmacologic target in neuroblastoma when knockdown of *ALK* resulted in growth inhibition in all neuroblastoma cell lines with *ALK* mutations and some with wild-type *ALK*.[25] Further testing with an *ALK* small molecule inhibitor, crizotinib, showed profound sensitivity *in vitro* and *in vivo* to the drug in a panel of neuroblastoma cell lines and xenografts, respectively, with certain mutations and *ALK* amplification.[56,58–60] Based on these data, only 18 months after *ALK* was discovered as a neuroblastoma oncogene, the Children's Oncology Group initiated a Phase I/II clinical trial testing crizotinib in patients with relapsed pediatric solid tumors and anaplastic large cell lymphoma (ALCL) (www.clinicaltrials.gov, Identifier: NCT00939770). Toxicity has remained low, and seven patients with ALCL and two patients with neuroblastoma have had complete responses as the trial continues.[61] This is a hallmark example of how identifying genetic susceptibility can be quickly advanced for clinical benefit.

However, there are some families that do not show mutations in *ALK* or *PHOX2B*, thus the search for additional familial neuroblastoma gene continues. Whole exome analysis of one family with two affected cousins and two healthy members showed a mutation in *GALNT14* predicted to be functionally damaging, but continued efforts are necessary to further define this familial variant.[113] In parallel, germline mutations in *TP53*, *SDHB*, *PTPN11*, *APC*, and *NFI* have been reported to occur rarely in neuroblastoma patients (Figure 1).[62–68,69] Neuroblastoma has also been reported to arise in complex congenital malformation syndromes, such as the subtelomeric 1p36.3 or 11q23 deletions.[70,71] The heritability of neuroblastoma remains only partially understood, yet continued investigation is expected to reveal new insights into familial neuroblastoma predisposition, including gene-gene and gene-environment interactions.

Genetic susceptibility to sporadic neuroblastoma

In familial neuroblastoma, there are rare mutations that lead to a high probability of disease. For the 99% of cases that occur sporadically, a common variant hypothesis proposes that common germline variations influence the probability of disease occurrence, each with a low relative risk, but presumably acting in concert. A large GWAS consisting of 720 neuroblastoma cases and 2,128 controls was undertaken in neuroblastoma as an unbiased method for discovering these polymorphisms (Figure 2).[89] This original GWAS has been expanded and replicated as additional patient samples have been accrued, leading to the identification of DNA alleles significantly associated with high-risk and low-risk neuroblastoma predisposition, including *CASC15*, *BARD1*, *LMO1*, *LIN28B*, *HACE1*, *DUSP12*, *DDX4*, *IL31RA*, *HSD17B12*, *NEFL*, *TP53*, AND *NBPF23* (Table 1).[72, 74, 75, 79, 81, 88, 89, 102–104, 107] The discovery of these susceptibility loci demonstrates the utility of interrogating GWAS signals for clues into the underlying biology driving neuroblastoma genesis.

Results from the initial GWAS identified three SNPs at chromosome 6p22 within a newly identified long noncoding RNA (lncRNA) annotated as *CASC15*. [72] Homozygosity for the risk alleles was significantly associated with metastatic disease, amplification of *MYCN* oncogene in the tumors, and patient relapse. Recently, decreased expression of the truncated isoform *CASC15-S* was associated with more advanced disease. [73] Another lncRNA, *NBAT-1* (*CASC14*), was shown to be located at the 6p22 susceptibility locus as well, and functional studies have shown that loss of *NBAT-1* promotes proliferation and invasion. [74] Subsequently, a GWAS restricted to high-risk neuroblastoma identified the BRCA-associated ring domain-1 gene (*BARD1*) at chromosome 2q35 was identified as a susceptibility locus.[75] Six SNPs were discovered in three different N-terminal introns of this gene. *BARD1*, along with its binding partner, breast cancer 1, early onset (*BRCA1*), had been previously implicated in breast and other cancers, but genetic variants in *BARD1* had not been shown to lead to cancer susceptibility, even in breast cancer.[76–78] Continuing efforts in *BARD1* have found that an isoform, BARD1 β , which lacks the RING domain necessary for BRCA1 binding, is preferentially expressed in neuroblastoma cell lines that are homozygous for the risk alleles.[79] Consistent with oncogenic behavior, knockdown of this isoform inhibits cell growth, while overexpression leads to increased proliferation. Additionally, BARD1 β was found to stabilize the Aurora family of kinases in neuroblastoma

cell lines, suggesting a possible mechanism of action and potential therapeutic strategy as Aurora kinase inhibitors are in clinical development for cancer.[79,80]

This GWAS was expanded (2,251 neuroblastoma cases and 6,097 controls) and the gene *LMO1* was shown to be significantly associated with high-risk neuroblastoma, which had previously been implicated in human cancer, but not neuroblastoma. Four SNPs that were significantly associated with neuroblastoma at chromosome 11p15.4 were within the LIM domain only 1 (*LMO1*) gene.[81] This gene, along with *LMO2*, *LMO3* and *LMO4*, encodes a cysteine-rich transcriptional cofactor that is preferentially expressed in the nervous system.[82] This family of genes has been found to be critically involved in leukemia (reviewed in ref. [83]) and breast cancers,[84–86] while *LMO3* has been shown to be oncogenic in neuroblastoma through its interaction with a neuronal-restricted transcription factor.[87] These common variations in *LMO1* were found to be associated with high-risk disease and decreased survival.[81] Neuroblastoma tumors with *LMO1* risk alleles were found to have increased expression of *LMO1*, and depletion of *LMO1* in cell lines decreased growth while forced over-expression increased growth.[81] This is consistent with a gain-of-function role in tumor progression. Recent investigation showed that the causal SNP resides in a super enhancer element within the first intron, with the G>A transversion ablating a canonical GATA transcription factor binding site. [88] Investigators showed that the A allele was “protective”, as there was no GATA binding, and not cis-mediated *LMO1* transcription, providing one of the first clear mechanistic insights into a genetic association.

By further expanding this GWAS to 2,817 neuroblastoma cases and 7,473 controls, two new association signals emerged at 6q16 in two different genes, *HACE1* and *LIN28B*. [89] *HACE1* encodes an E3 ubiquitin ligase and has been identified as a tumor suppressor gene silenced in Wilms’ tumors, colorectal cancer, and gastric carcinoma.[90–92] It has also been shown to suppress cell growth in human cancer cells, including a neuroblastoma cell line, by inhibiting cell cycle progression during stress.[93] *LIN28B*, a known oncogene, encodes an RNA-binding protein that is developmentally regulated and blocks the expression of the let-7 family of microRNAs.[94] High expression of *LIN28B* and correlated low levels of let-7 have been observed in many human cancers.[95,96] *LIN28B* and let-7 are involved in stem cell differentiation, as overexpressing the former or inhibiting the latter leads to the reprogramming of human and mouse fibroblasts into pluripotent stem cells.[97,98] In the GWAS, *LIN28B* was expressed at significantly higher levels in neuroblastoma cell lines homozygous for the risk allele, and this correlated with lower levels of let-7 and growth inhibition following knockdown of *LIN28B*. [89] In tumor samples, *HACE1* expression was significantly lower and *LIN28B* significantly higher in high-risk neuroblastomas and were correlated similarly with worse overall survival. Mechanistic studies have shown that *LIN28B* promotes increased expression of the oncogenic protein RAN, which both converge on Aurora Kinase A.[99] Increased activity was shown to drive tumorigenesis, providing further evidence that targeting Aurora kinases may provide a benefit to neuroblastoma patients.[100,101]

In an integrated proteomic-GWAS approach, Capasso identified three SNPs significantly associated with neuroblastoma in the *NEFL* gene, encoding the light chain neurofilament protein in which mutations are known in disorders of the peripheral nervous system.[102]

Overexpression of *NEFL* in cells with a protective allele caused cells to adopt a more differentiated phenotype and to have reduced proliferative capacity. The authors suggested that decreased expression of *NEFL* alters the differentiation state of sympathetic neurons and may predispose neuroblastoma.[102]

After enriching the GWAS for patients with low-risk neuroblastoma, SNPs in four genes, *DUSP12*, *DDX4*, *IL31RA* and *HSD17B12*, were discovered to be significantly associated with this phenotypic subset.[103] These genes are different than those found in high-risk neuroblastoma, suggesting these subtypes are likely genetically distinct and emphasizing the importance of robust phenotypic information in GWAS efforts. These data further support the notion that widely divergent neuroblastoma phenotypes are genetically predetermined.

A genome wide SNP scan for copy number variation (CNV) identified a novel CNV at 1q21.1 that is associated with neuroblastoma, and they were able to confirm deletions in this region by quantitative PCR and FISH.[104] A new neuroblastoma breakpoint family gene, *NBPF23*, was identified at this location by a transcript that was similar to other genes in the family. This transcript is most commonly expressed in fetal brain and sympathetic nervous system tissues, and in neuroblastoma, its expression was correlated with this CNV. *NBPF1* was identified originally at the translocation breakpoint in the germline of a child with neuroblastoma,[68] and research continues to elucidate the role of this family of genes in disease development.

The prevalence of GWAS-associated genes has been further interrogated among different ethnic groups. A follow up study to the previously described *BARD1* GWAS was carried out in African American children with neuroblastoma looking at SNPs in the gene regions identified by the GWAS in Caucasians.[106] Two of the six SNPs found in *BARD1* were also significantly associated with neuroblastoma in the African-American cohort, validating the original GWAS. Due to different patterns of linkage disequilibrium in the two ethnicities, this effort narrowed the potential location of the causal variant. Another study in patients of African descent identified an allele in a new gene, sperm associated antigen 16 (*SPAG16*), associated with high-risk neuroblastoma in patients of both African and European ancestry showing the potential of discovering new associations by studying specific ethnic groups. [107]

In the Oldridge manuscript noted above defining a mechanistic basis for the LMO1 association, the protective T-allele was noted to be common in people of European ancestry, but is largely absent in African and African-American populations, which retain the G-allele. [102] This may provide a partial explanation for the more aggressive forms of neuroblastoma observed in African-American patients. Altogether, these results indicate that ethnic background may play a role in genetic predisposition and that therapeutic approaches may require requisite tailoring.

Collectively, these GWAS-discovered genes account for only a small portion of neuroblastoma heritability, which remains poorly understood. It is likely that further expansion of GWAS efforts will continue to uncover more susceptibility genes that will confer risk in an additive manner. No epistasis was found when the most significant SNPs

from 2q35, 6p22, 11p15.4 and 1q21.1 CNV were studied together;[81] however, specific clusters of combinations of these SNPs were significantly associated with neuroblastoma. [105] Mechanistic insights are being discovered, but the underlying basis for most statistical associations remain unknown. Neuroblastoma GWASs were expected to discover genes that affect development of the sympathetic nervous system, showing that common variants can lead to missteps in development and therefore malignancies. Investigators are pursuing ongoing studies to model GWAS variants and heritability in zebrafish and induced pluripotent stem cell models to understand the biological consequences in neuroblastoma and investigate potential therapeutic interventions.

Rare Variants

There are currently two main groups of germline DNA variations that predispose to neuroblastoma: very rare genetic mutations leading to Mendelian inheritance of familial neuroblastoma with a high penetrance, and common variations that only increase risk of disease in small increments. These discoveries thus far have only explained a small proportion of the heritability of neuroblastoma. While further expansion of the GWAS will continue to uncover more common variants and genes important in the development of neuroblastoma, we suggest that these discoveries lie on a spectrum with the middle ground only beginning to be realized (Figure 1). These are rare germline variations or mutations with a lower penetrance than familial disease but with a larger effect on predisposition than the common SNPs. Owing to their rarity and the relatively small number of patients with neuroblastoma, it has been difficult to identify these rare variants. Recently, two rare germline variants in *TP53* were found to be robustly associated with neuroblastoma using the 1000 Genomes Project[108] and an advanced imputation process elucidating associations with SNPs not directly assayed on the limited arrays.[109] Likewise, germline sequencing has identified putative damaging mutations in *ALK*, *CHEK2*, *PINK1*, *BARD1* and *APC1* in small percentages of patients with neuroblastoma.[69,110]. As sequencing technology improves and costs decrease, discoveries of additional rare variants are on the horizon to define and characterize further the heritability of neuroblastoma. The influence of germline mosaicism and epistatic interaction of de novo or inherited mutations with GWAS-defined polymorphisms remains undefined.

Summary and Future Directions

Significant progress has been made in the last six years in describing the genetic landscape of neuroblastoma and continuing studies will aim to further identify Mendelian susceptibility genes. This is already influencing clinical care as genetic testing is available, and there are noninvasive screening methods to surveil for disease in young children. Current recommendations suggest that children with a known damaging germline mutation in *ALK* or *PHOX2B* based on familial pedigrees should undergo surveillance with every 3-month ultrasonography and urinary catecholamines until a minimum of age 5, if not beyond. [111] The main impact of GWAS studies to date is in identifying genes critical to neuroblastoma progression and maintenance, thus uncovering potential oncogenic vulnerabilities. With the discovery of *ALK* as an example, it is important that translational approaches related to these genes be prioritized, as additional targeted therapies for patients

with neuroblastoma are essential to improving survival. Future work to extend the discovery of germline polymorphisms to those that influence response to therapy and impact comorbidities such as hearing loss also has the potential to improve patient survival and quality of life. The ultimate goal of genomic studies in neuroblastoma is to inform precision medicine with genetic evaluations to tailor clinical treatments and extend survival.[113] As additional patient samples are accrued over time, future GWAS endeavors will be required to continue the discovery of additional susceptibility alleles. Extensive further investigation, both computationally and in designing better models for these rare genetically defined subsets, will be required to translate these genomic discoveries into actionable targets for diagnosis and treatment.

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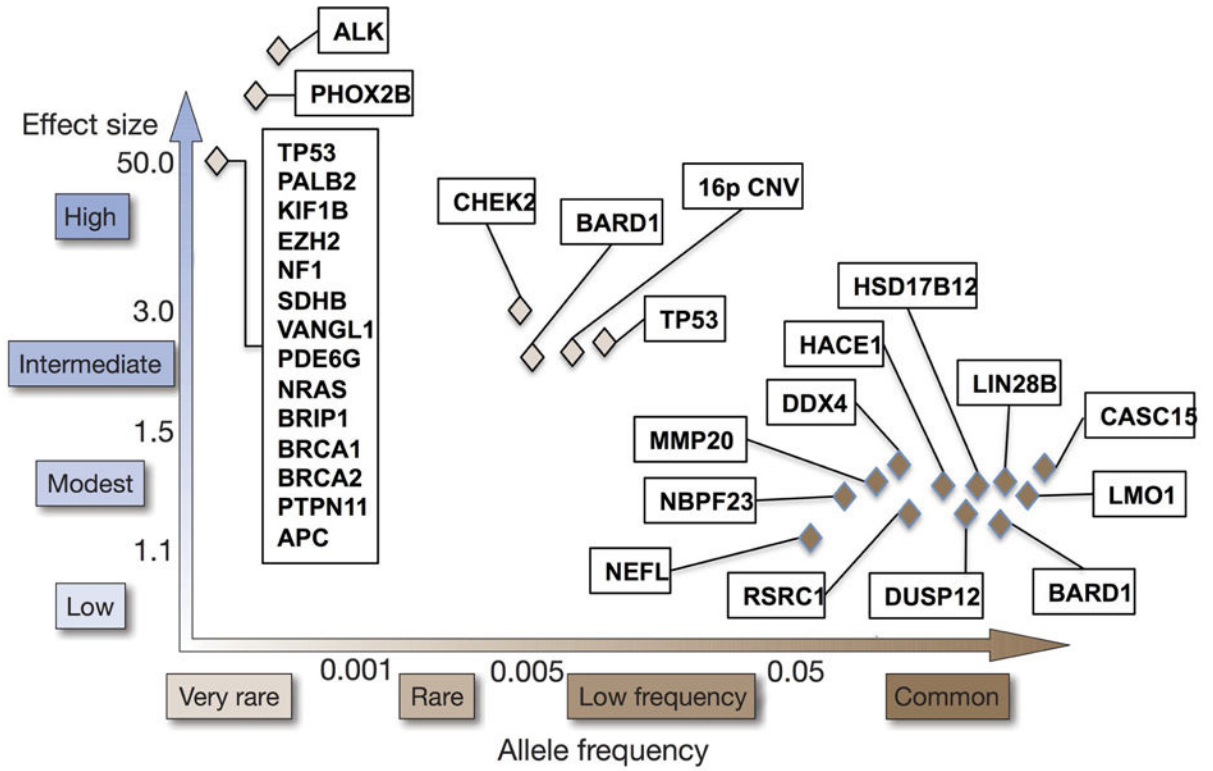


Figure 1. Graphical representation of genetic predisposition to neuroblastoma. Known familial and sporadic predisposition genes have been compiled into one summary figure across multiple studies. The familial mutations are shown in the top left of the graph representing a very rare allele frequency and high effect size. GWAS-discovered variations are in the bottom right corner representing a higher allele frequency with a lower effect size. Continued sequencing efforts are likely to uncover additional rare susceptibility variants along this spectrum, of which dozens are predicted to be discovered to explain the heritability of neuroblastoma.

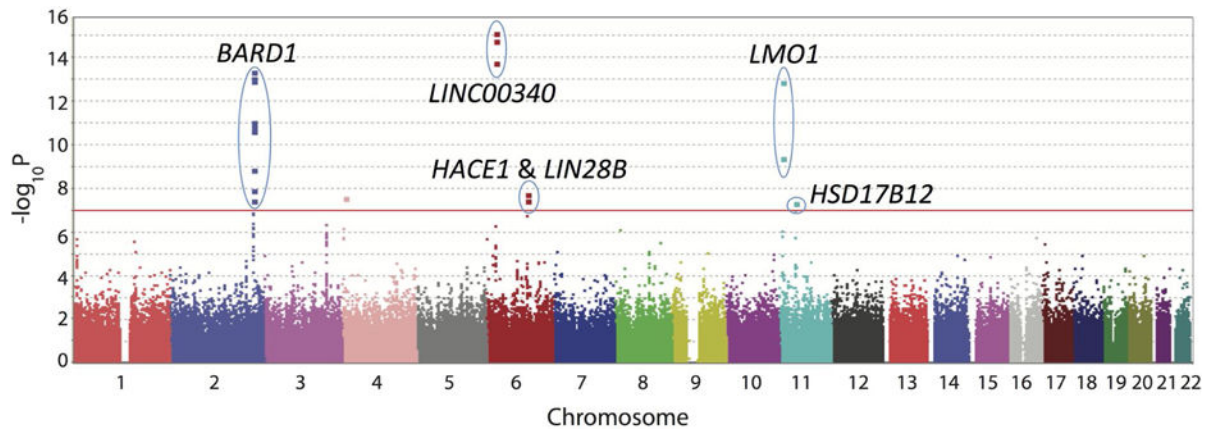


Figure 2.

Manhattan plot of high-risk neuroblastoma GWAS results across multiple studies. Level of significance ($-\log_{10}$ transformed p values) for each SNP along the genome in chromosomal order is plotted, and the corresponding genes are labeled. Red line: genome-wide significance threshold based on Bonferroni adjustment. Adapted from Diskin, et al. 2012.

Summary of neuroblastoma susceptibility loci. A majority of this cohort of genomic loci are significantly associated with distinct neuroblastoma phenotypes, while some remain to be characterized. *P* values and Odds Ratios (ORs) are combined values between discovery and replication studies from the original publication. Predicted mechanisms on protein function are indicated as loss of function, gain of function, or currently unknown. MAF = minor allele frequency.

Table 1

Genomic Locus	Phenotype Association	Top SNP	<i>P</i> -Value (combined)	MAF cases	Odds Ratio (OR)	Proposed Mechanism
6p22	High-risk	rs6939340	9.33×10^{-15}	56%	1.37	Loss of function
2q35	High-risk	rs6435862	5.20×10^{-18}	40%	1.68	Gain of function
11p15	High-risk	rs110419	5.20×10^{-16}	55%	1.34	Gain of function
6q16	High-risk	rs17065417	1.20×10^{-8}	8%	1.38	Gain of function
6q16	High-risk	rs4336470	2.70×10^{-7}	30%	1.26	Loss of function
1q23	Low-risk	rs1027702	2.07×10^{-6}	31%	2.01	unknown
5q11	Low-risk	rs2619046	2.94×10^{-6}	32%	1.47	unknown
5q11	Low-risk	rs10055201	6.54×10^{-7}	29%	1.49	unknown
11p11	Low-risk	rs11037575	4.20×10^{-7}	39%	1.67	unknown
8p21		rs11994014	0.005	20%	0.88	Loss of function
17p13		rs35850753	3.34×10^{-14}	3.6%	2.7	Loss of function
1q21		CNV	2.97×10^{-17}	15%	2.49	unknown