Advances in the Translational Genomics of Neuroblastoma: From Improving Risk Stratification and Revealing Novel Biology to Identifying Actionable Genomic Alterations

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Neuroblastoma is an embryonal malignancy that commonly affects young children and is remarkably heterogeneous in its malignant potential. Recently, the genetic basis of neuroblastoma has come into focus and not only has catalyzed a more comprehensive understanding of neuroblastoma tumorigenesis but also has revealed novel oncogenic vulnerabilities that are being therapeutically leveraged. Neuroblastoma is a model pediatric solid tumor in its use of recurrent genomic alterations, such as high-level MYCN (v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog) amplification, for risk stratification. Given the relative paucity of recurrent, activating, somatic point mutations or gene fusions in primary neuroblastoma tumors studied at initial diagnosis, innovative treatment approaches beyond small molecules targeting mutated or dysregulated kinases will be required moving forward to achieve noticeable improvements in overall patient survival. However, the clonally acquired, oncogenic aberrations in relapsed neuroblastomas are currently being defined and may offer an opportunity to improve patient outcomes with molecularly targeted therapy directed toward aberrantly regulated pathways in relapsed disease. This review summarizes the current state of knowledge about neuroblastoma genetics and genomics, highlighting the improved prognostication and potential therapeutic opportunities that have arisen from recent advances in understanding germline predisposition, recurrent segmental chromosomal alterations, somatic point mutations and translocations, and clonal evolution in relapsed neuroblastoma.


KEYWORDS: anaplastic lymphoma kinase (ALK), clonal evolution, genome-wide association studies, neuroblastoma, pediatric, v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog (MYCN).

INTRODUCTION

Neuroblastoma is an embryonal tumor that arises from the aberrant growth of neural crest progenitor cells of the developing sympathetic nervous system. Tumors typically arise in the adrenal medullary tissue or paraspinal sympathetic ganglia and present as masses in the abdomen, chest, or neck.1 Neuroblastoma occurs in very young children, with a median age of 17 months at diagnosis.2 It is the most frequent malignancy during infancy, accounting for >20% of cancers diagnosed during the first year of life, and it can be detected in utero.2,3 Although neuroblastoma constitutes only approximately 5% of all pediatric cancer diagnoses, it disproportionately causes up to 10% of childhood cancer mortality.4

A hallmark of neuroblastoma is its heterogeneity in clinical presentation, course, and overall prognosis, ranging from infants with tumors that can spontaneously regress; to children who have localized tumors with favorable genomic characteristics and excellent overall survival with limited cytotoxic chemotherapy; to critically ill older children, adolescents, or young adults with widely disseminated disease that can grow relentlessly despite intensive multimodal chemoradiotherapy.5,5,6 Although remarkable improvements over the last 3 decades have been made for children with lower stage disease, children with high-risk neuroblastoma continue to often have incurable disease with survival rates of <40% despite dramatic escalations in the intensity of cytotoxic chemoradiotherapy.1,5,7,9

Neuroblastoma serves as a model cancer for the clinical utility of comprehensive characterization of tumor genetic and biologic features at the time of diagnosis. The discovery of frequent, high-level amplification of the v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog (MYCN) oncogene on chromosome 2p24 in neuroblastosoma and its association with aggressive clinical disease in the 1980s ushered in the era of using prognostic genomic biomarkers for neuroblastoma and, arguably, for cancer in general.10,11 Risk stratification in neuroblastoma has evolved...
over time to encompass both easily measured clinical variables, such as patient age and tumor stage, and precise genomic aberrations that have been identified as predictive of clinical outcome, such as DNA ploidy and specific recurrent segmental chromosomal aberrations.12-16 A multinational collaborative effort was initiated in 2004 to develop a standardized International Neuroblastoma Risk Group (INRG) classification system.17,18 A pooled cohort of 8800 patients treated over 12 years was analyzed considering 13 potential prognostic factors.17 Four broad prognostic clinical categories were identified (very low risk, low risk, intermediate risk, and high risk) from analyses of the 7 most significant prognostic factors at the time of tumor diagnosis: age at diagnosis, INRG tumor stage, tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status at tumor histologic category, grade of tumor differentiation, DNA ploidy, and copy-number status.

NEUROBLASTOMA GENETIC PREDISPOSITION

Neuroblastoma, like other pediatric embryonal malignancies, has long been thought to arise from the malignant progression of misappropriated fetal tissue during development. However, it was not until this past decade that we gained a comprehensive understanding of the underlying genetic events driving neuroblastoma tumorigenesis.19-34 Recently, many of the initiating genetic events for both familial and sporadic neuroblastoma have been defined; and not only has this led to an improved understanding of the transforming biologic aberrations that contribute to neuroblastoma tumorigenesis, but these investigations have also identified potentially druggable, activated pathways that have been translated into advances in the clinic.19-38 In part, the persistence of the effects of these oncogenes in established tumors may explain the paucity of recurrent, somatic oncogenic driver mutations identified in recent whole-genome sequencing efforts.39-42

FAMILIAL NEUROBLASTOMA

Similar to other human malignancies, a small subset of neuroblastoma is inherited in an autosomal-dominant manner; and, in these families, the disease generally occurs at a younger age and is more likely to present with multifocal primary tumors than sporadic tumors, as predicted by Knudsen’s 2-hit hypothesis.43,44 Familial neuroblastoma is rare, only accounting for 1% to 2% of neuroblastoma cases, and gain-of-function mutations in the anaplastic lymphoma kinase (ALK) gene were recently identified as the major cause of highly penetrant, hereditary disease.22,24 ALK is an orphan (ligand not known) receptor tyrosine kinase that acts as a major oncogenic driver in several human malignancies, including anaplastic large cell lymphoma and non–small cell lung carcinoma; however, unlike in neuroblastoma, in these malignancies, ALK is typically activated by translocation events, which create aberrantly expressed fusion transcripts that contribute to cellular transformation and progression.38 In familial neuroblastoma, constitutive ALK activation is achieved through kinase domain mutations, and often identical activating mutations also have been identified in sporadic neuroblastoma tumors.22,24,36-42,45 However, although the most common ALK germline mutation is also the most common somatic ALK alteration (an arginine-to-glutamine substitution at codon 1275 [R1275Q]), the most activating somatic ALK variants typically do not appear to be tolerated in the germline.45,46 In the rare cases that the more potent ALK mutations (phenylalanine-to-valine substitutions at codons 1245 and 1174 [F1245V and F1174V, respectively]) have appeared in the germline, they result not only in congenital neuroblastoma but also in global developmental and neurocognitive defects, suggesting an important role for ALK regulation in the normal development of the central nervous system.45,46 Furthermore, the presence of ALK-activating mutations in the germline appears to be directly correlated with the level of aberrant kinase activation.45 On the basis of these preclinical findings, small-molecule inhibitors of ALK kinase activity have been translated rapidly, albeit with extensive and thorough preclinical study, to neuroblastoma patient care in early phase clinical trials.47

Finally, a small subset of familial and sporadic neuroblastomas that typically occur along with other anomalies of neural-crest derived tissues is caused by loss-of-function mutations in the paired-like homeobox 2B (PHOX2B) gene on chromosome 4p12 that encodes a transcription factor integral to neural crest development, loss of which disrupts terminal differentiation of neuroblastoma cells.48 PHOX2B mutations are identified in a majority of children with congenital central hypoventilation syndrome and in some children with Hirschsprung disease, but they account only for approximately 10% of
familial neuroblastomas. Genetic testing for germline ALK or PHOX2B mutations has become the standard of care for children with a family history of neuroblastoma and also should be considered for children who have bilateral adrenal tumors. Typically, asymptomatic children who have an ALK or PHOX2B mutation identified are generally screened with serial abdominal ultrasound studies and urine catecholamine levels approximately every 3 months until age 5 years. However, currently, there are no data or consensus guidelines to support these clinical practices, and there is an urgent need to address this prospectively. Ongoing efforts are focused on discovering the cause of the remaining 10% to 20% of familial neuroblastomas that do not harbor obvious ALK or PHOX2B mutations.

**SPORADIC NEUROBLASTOMA PREDISPOSITION**

We now know that sporadic (nonfamilial) neuroblastoma is a complex genetic disease in which common polymorphic alleles contribute substantively to malignant transformation. A large genome-wide association study (GWAS) of samples that were collected by the cooperative efforts of the Children’s Oncology Group using high-density single nucleotide polymorphism (SNP)-based microarray technology has begun to define the genetic landscape of sporadic neuroblastoma predisposition and have identified common DNA alleles in cancer susceptibility candidate 15 (nonprotein coding) (CASC15), BRCA1 associated RING domain 1 (BARD1), LIM domain only 1 (LMO1), dual specificity phosphatase 12 (DUSP12), DEAD (Asp-Glu-Ala-Asp) box polypeptide 4 (DDX4), interleukin 31 receptor A (IL31RA), hydroxysteroid (17β) dehydrogenase 12 (HSD17B12), HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1 (HACE1), lin-28 homolog B (LIN28B), and neurofilament, light polypeptide (NEFL) that are associated significantly with neuroblastoma development (Fig. 1, Table 1).

Although the motivation for the study was to discover neuroblastoma-initiating events, it has become clear that many of the neuroblastoma GWAS loci harbor driver oncogenes that are critical in maintaining tumorigenicity in established tumors. These discoveries have collectively illustrated how robust GWAS signals offer genomic landmarks that point toward the identification of molecular mechanisms involved in both tumor initiation and malignant progression and, in some instances, offer insights into druggable biologic pathways that may be leveraged clinically (Fig. 1, Table 1).

In that GWAS, one of the most significant and robustly replicated association signals that was enriched in the high-risk subset of neuroblastomas resided in the BARD1 locus on chromosome 2q35. BARD1 has been classically thought of as a tumor suppressor, because its protein dimerizes with BRCA1 through their respective RING domains. However, in neuroblastoma, it was discovered that disease-associated variations were correlated with the expression of an oncogenetically activated isoform, BARD1β, which has growth-promoting effects in...
TABLE 1. Summary of Genomic Loci Significantly Associated With Neuroblastoma Predisposition

<table>
<thead>
<tr>
<th>Genomic Locusa</th>
<th>Candidate Gene</th>
<th>Phenotype Association</th>
<th>Top SNP</th>
<th>P (Combined)b</th>
<th>MAF Cases, %</th>
<th>OR</th>
<th>Proposed Mechanism</th>
<th>Level of Evidence (References)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>2q35</td>
<td>BARD1</td>
<td>High risk</td>
<td>rs6435862</td>
<td>8.65 × 10⁻¹⁸</td>
<td>40</td>
<td>1.68</td>
<td>Gain of function</td>
<td>2 (Capasso 2009, 21 Bosse 2012, 22)</td>
</tr>
<tr>
<td>11p15</td>
<td>LMO1</td>
<td>High risk</td>
<td>rs110419</td>
<td>5.20 × 10⁻¹⁶</td>
<td>55</td>
<td>1.34</td>
<td>Gain of function</td>
<td>1 (Wang 2011, 26 Oldridge 2015, 32 2016)</td>
</tr>
<tr>
<td>6q16</td>
<td>LINC28</td>
<td>High risk</td>
<td>rs1706547</td>
<td>1.2 × 10⁻⁸</td>
<td>8</td>
<td>1.38</td>
<td>Gain of function</td>
<td>1 (Diskin 2012, 27 Molenaar 2012, 33 2012)</td>
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<tr>
<td>6q16</td>
<td>HACE1</td>
<td>High risk</td>
<td>rs4336470</td>
<td>2.7 × 10⁻⁶</td>
<td>30</td>
<td>1.26</td>
<td>Loss of function</td>
<td>3 (Diskin 2012, 27 Molenaar 2012, 33 2012)</td>
</tr>
<tr>
<td>1q23</td>
<td>DUSP12</td>
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<td>rs1027702</td>
<td>2.07 × 10⁻⁶</td>
<td>31</td>
<td>2.01</td>
<td>Unknown</td>
<td>4 (Nguyen, 34)</td>
</tr>
<tr>
<td>5q11</td>
<td>DDX4</td>
<td>Low risk</td>
<td>rs2619046</td>
<td>2.94 × 10⁻⁶</td>
<td>32</td>
<td>1.47</td>
<td>Unknown</td>
<td>4 (Nguyen, 34)</td>
</tr>
<tr>
<td>5q11</td>
<td>ILS1RA</td>
<td>Low risk</td>
<td>rs10065202</td>
<td>6.54 × 10⁻⁷</td>
<td>29</td>
<td>1.49</td>
<td>Unknown</td>
<td>4 (Nguyen, 34)</td>
</tr>
<tr>
<td>11p11</td>
<td>HSD17B12</td>
<td>Low risk</td>
<td>rs11037575</td>
<td>4.20 × 10⁻¹⁷</td>
<td>39</td>
<td>1.67</td>
<td>Unknown</td>
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</tr>
<tr>
<td>8p21</td>
<td>NEFL</td>
<td>—</td>
<td>rs11994014</td>
<td>.005</td>
<td>20</td>
<td>0.88</td>
<td>Loss of function</td>
<td>2 (Capasso 2014, 35)</td>
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<td>17p13</td>
<td>TP53</td>
<td>—</td>
<td>rs85850753</td>
<td>3.43 × 10⁻¹⁴</td>
<td>3.6</td>
<td>2.70</td>
<td>Loss of function</td>
<td>1 (Diskin 2014, 36 Schliefermacher 2012, 37 2012)</td>
</tr>
<tr>
<td>1q21</td>
<td>NBPFF23</td>
<td>—</td>
<td>CNV</td>
<td>2.97 × 10⁻¹⁷</td>
<td>15</td>
<td>2.49</td>
<td>Unknown</td>
<td>4 (Diskin 2009, 38)</td>
</tr>
</tbody>
</table>

Abbreviations: BARD1, BRCA1 associated RING domain 1; CASC15/NTB-1, cancer susceptibility candidate 15 (nonprotein coding)/neuroblastoma associated transcript 1; CNV, copy number variant; DDX4, DEAD (Asp-Glu-Ala-Asp) box polypeptide 4; DUSP12, dual specificity phosphatase 12; HACE1, HECT domain and ankyrin repeat containing E3 ubiquitin ligase 1; HSD17B12, hydroxysteroid (17beta) dehydrogenase 12; ILS1RA, interleukin 31 receptor A; LIN28B, lin-28 homolog B; LMO1, LIM domain only 1; MAF, minor allele frequency; NEPL, neuroblastoma breakpoint family, member 23; NEFL, neurofilament, light polypeptide; OR, odds ratio; p, short arm; q, long arm; rs, reference single nucleotide polymorphism; SNP, single nucleotide polymorphism; TP53, tumor protein 53.

aMultiple genomic loci are associated with a specific neuroblastoma clinical phenotype as detailed and have strong functional evidence linking the associated genes to neuroblastoma genesis, whereas others have functions that have yet to be explored in neuroblastoma.
bP values and odds ratios are based on combined results from discovery and replication cohorts from the original publication, as indicated.
cFor levels of evidence, 1 indicates strong functional/mechanistic evidence of being a neuroblastoma associated gene; 2, some functional/mechanistic evidence in neuroblastoma, 3, some functional/mechanistic evidence in other malignancies; 4, biologic function in neuroblastoma/other malignancies unknown.

neuroblastoma models potentially through cooperation with the Aurora family of kinases.32 In that study, not only did we identify BARD1 as an oncogenic driver of high-risk neuroblastoma tumorigenesis, but we also reidentified a potential druggable pathway in neuroblastoma, because Aurora A kinase inhibitors were independently identified as effective therapy in neuroblastoma models.56 Although the common polymorphisms at the BARD1 locus associated with neuroblastoma have a modest impact individually on disease initiation, it is now evident that rare germline mutations in this gene also contribute to tumorigenesis, likely with a much higher effect size (Fig. 1).40 However, whether or not these patients are also susceptible to breast and/or ovarian cancer and identifying the functional consequences and penetrance of these mutations will require further study.

LMO1 is another neuroblastoma oncogene that was discovered through the GWAS described above; and, again, we observed that this association was enriched in the high-risk phenotype.20 In other words, individuals with risk alleles in the BARD1 and LMO1 loci are not only more likely to develop neuroblastoma; however, if the disease does develop, then they are also much more likely to develop metastatic disease and have worse clinical outcome probabilities. This concept—that the ultimate clinical phenotype when patients present with neuroblastoma is largely dictated by underlying germline genotypes—is yet another unexpected conclusion from the GWAS. LMO1 is a transcription regulator and is the first example in neuroblastoma in which a definitive disease-causal SNP has been identified. Briefly, a single G>T transversion in the first intron of LMO1 was discovered in a super-enhancer element, and the G (risk) allele allows for GATA3 transcription factor binding, leading to robust LMO1 expression.20,34 The T (protective) allele completely abrogates GATA3 binding, resulting in dramatically lower LMO1 expression. Many other robust neuroblastoma association signals have been discovered from this GWAS that collectively can be estimated to have a combined odds ratio close to 20 for developing this disease, and functional follow-up studies have confirmed that many of these genes are also driver genes in established tumors (Fig. 1, Table 1).19-21,23,25-34,56

In addition to common SNP variation, neuroblastoma was the first example in which germline copy number variation, another major determinant of human diversity, contributes to cancer susceptibility.30 Neuroblastoma breakpoint family, member 23 (NBPFF23) is a gene on chromosome 1q21.1 that harbors a region that varies in copy number among individuals, and hemizygous deletion of this region is highly associated with neuroblastoma. Other yet to be discovered copy number
Variations in TP53 are also likely to contribute to neuroblastoma tumorigenesis.

Finally, because this GWAS has continued to accrue participants, it has become statistically feasible to identify rare polymorphisms that contribute to neuroblastoma susceptibility (Fig. 1, Table 1). For example, rare germline variants in tumor protein 53 (TP53) have also been identified as integral to neuroblastoma genesis.26 Recently, 2 reference SNPs, rs78378222 and rs35850753, which map to the 3'-untranslated region (UTR) and the 5'-UTR of TP53, respectively, were robustly associated with neuroblastoma predisposition.26 Similar to other malignancies, the disease-associated rs7878222 allele is hypomorphic and impairs transcriptional termination and polyadenylation of TP53 transcripts.26,57 However, the functional implications of the other noncoding SNP, rs35850753, remain unclear but may involve driving TP53 alternative transcript expression, because it maps to the 5'-UTR of the 6133 TP53 isoform, which is transcribed off of an alternative, internal promoter. This isoform lacks a TP53 transactivation domain and has demonstrated the ability to acts in a dominant-negative fashion to the tumor-suppressive functions of wild-type TP53.59 Because somatic TP53 mutations are rare in neuroblastoma, these results suggest that alternative mechanisms may be used by neuroblastoma cells to circumvent the tumor-suppressor effects of TP53 in the development of a malignant phenotype.39-42,60

**SOMATIC GENOMIC ABERRATIONS IN NEUROBLASTOMA: PROGNOSTIC IMPLICATIONS AND THERAPEUTIC OPPORTUNITIES**

The discovery of high-level MYCN amplification on chromosome 2p24 and its association with aggressive clinical disease in the 1980s heralded the beginning of using tumor-specific genomic alterations as prognostic biomarkers of neuroblastoma10,11 and, arguably, of cancer in general. Since then, several other recurrent genomic alterations have been robustly validated as having prognostic predictive power in addition to MYCN amplification, including DNA ploidy, gain of chromosome 17q, and deletions of chromosome arms 1p, 3p, 4p, or 11q.12-17,61-66 However, although gains or losses of these somatic loci are clinically prognostic in neuroblastoma, the candidate oncogenes or tumor-suppressor genes at these loci that underlie this difference in tumor biology have yet to be conclusively defined.

**CHROMOSOMAL REARRANGEMENTS**

The ploidy (DNA index) of neuroblastoma primary tumors has become a significant predictor of patient outcome and is a main component of the current INRG classification system.17 Diploid tumors (DNA index = 1) occur in approximately 33% of patients, whereas 66% of neuroblastoma tumors are hyperdiploid (DNA index > 1).15 Tumor ploidy is one of the most significant prognostic markers for children younger than 18 months, and infants with hyperdiploid tumors have significantly increased survival.15-17 Because determining the tumor DNA index is straightforward, it continues to be used as a prognostic marker by most cooperative groups. However, with the advent of next-generation genomic techniques, it has become clear that neuroblastoma tumor DNA copy number aberrations can be more precisely segregated into 2 main global categories that, independent of the specific genomic loci involved, carry clinically prognostic information.61,67 In general, tumors with gains of whole chromosomes resulting in hyperdiploidy are associated with lower risk disease and favorable clinical outcomes, whereas tumors that have segmental chromosomal alterations are associated with higher risk and more clinically aggressive disease.61,67 In addition, the acquisition of new segmental chromosomal aberrations appears to occur at the time of relapse in tumors that both did and did not have a segmental genomic profile at diagnosis.68 Undoubtedly, prospective studies are needed to evaluate whether the presence of segmental chromosomal aberrations at the time of diagnosis or relapse can be prognostically useful. It would be of great clinical utility if the presence of segmental chromosomal aberrations could identify the rare, less favorable subsets of patients within non-high-risk neuroblastoma (ie, those who have localized disease without MYCN amplification) who are predetermined to relapse or progress after surgery, enabling the upfront initiation of adjuvant cytotoxic chemotherapy.68,69 One recent clinical trial that has begun to address these clinical questions is the European Low and Intermediate Risk Neuroblastoma Study (LINES) 2009 (clinicaltrials.gov identifier NCT01728155).

Although segmental chromosomal aberrations are common in neuroblastoma, translocation events that create fusion oncogenes are rarely observed in diagnostic neuroblastomas, but they do occur more frequently in patients who have relapsed disease after exposure to intensive DNA-damaging agents. It has been demonstrated that structural rearrangements in protein-coding genes occur in primary neuroblastomas at an average of 4 per primary tumor,39 and up to approximately 20% of
predicted fusion transcripts can be detected at the RNA transcript level in neuroblastoma, including fusions transcripts involving the ALK, neuroblastoma amplified sequence (NBAS), protein tyrosine phosphatase receptor type D (PTPRD), and odd Oz/ten-m homolog 4 (ODZ4) genes. However, although ALK rearrangements leading to a constitutively active ALK fusion gene product are common drivers of the pathogenesis of non–small cell lung cancer and pediatric anaplastic large cell lymphoma, overall, they are rare in neuroblastoma tumors. Over the coming decade, next-generation sequencing technologies coupled with follow-up functional studies should allow for a more comprehensive profiling of the role that genomic structural arrangements and resulting fusion genes play in driving neuroblastoma tumorigenesis. Finally, chromothripsis, or the local shredding of chromosomal structural arrangements and resulting fusion events coupled with follow-up functional studies should allow for a more comprehensive profiling of the role that genomic structural arrangements and resulting fusion genes play in driving neuroblastoma tumorigenesis. Chromothripsis, or the local shredding of chromosomes, may be common in higher stage neuroblastomas. Although some studies have indicated that this occurs in up to 18% of clinically aggressive tumors, chromothripsis was not as common in other cohorts of primary neuroblastomas. Chromothripsis is in primary neuroblastomas awaits additional next-generation sequencing efforts.

CHROMOSOMAL ALLELIC GAINS AND ONCOGENES

The classic genetic aberration of neuroblastoma most consistently associated with a poor outcome is genomic amplification of the MYCN oncogene on chromosome 2p24. MYCN amplification occurs in approximately 20% of all primary neuroblastoma tumors and in 50% of high-risk tumors, and it correlates strongly with clinically aggressive, advanced-stage disease and treatment failure. Transgenic mice that are engineered to overexpress MYCN in their neuroectodermal cells developed neuroblastoma, providing a direct link of this gene to neuroblastoma genesis. MYCN encodes a transcription factor that forms heterodimers with MAX (a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with MYCN); and, together, they collectively activate the transcription of downstream target genes. RNA interference-induced knockdown of MYCN decreases neuroblastoma tumorigenesis by inducing apoptosis and terminal differentiation, suggesting that the direct targeting of this protein may be an effective therapeutic strategy. However, targeting MYCN with small-molecule inhibitors has proven to be challenging, in part because of difficulties targeting ubiquitously expressed, pleiotropic transcription factors, a hurdle that is further exacerbated by the limited small-molecule–binding real estate of MYCN among its 2 extended α helices. Thus, although MYCN amplification has been associated with aggressive neuroblastoma for over 30 years, it was not until recently that targeting this protein in neuroblastoma became a therapeutic reality through the development of novel therapeutic strategies to circumvent the requirement of direct MYCN interaction. These strategies include blocking the MYCN/MAX interaction, the development of small molecules that disrupt MYCN transcription or stability of the MYCN protein, and synthesizing drugs that target synthetic, lethal interactions of MYCN (for reviews, see Barone et al and Huang and Weiss).

One such strategy that holds therapeutic promise is the use of bromodomain and extra terminal (BET) inhibitors to specifically down-regulate MYCN expression. The family of BET adaptor proteins (BRD2-4) have bromodomain motifs that enable binding to histone tail acetylated lysine residues at sites of open chromatin, recruiting additional chromatin-modifying proteins and, thus, modulating target gene transcription. Small molecules targeting the bromodomains of these chromatin readers inhibit BET recruitment to open chromatin sites and, thus, decrease target gene transcription. Among the genes targeted by the BET family of chromatin readers are the MYC family genes. Screening of over 673 well characterized human cancer cell lines from the Cancer Cell Line Encyclopedia using the prototypical BET inhibitor JQ1 identified MYCN amplification as a robust biomarker for sensitivity to bromodomain inhibition; and, not surprisingly, bromodomain inhibition was cytotoxic both in neuroblastoma cell lines and in several neuroblastoma mouse models. Similar cell cytotoxicity has been observed with other BET inhibitors by the blockade of MYCN expression, and BET inhibitors were identified as efficacious in another pediatric embryonal malignancy, medulloblastoma, a subset of which is driven by MYC. Clearly, bromodomain inhibition may be a therapeutic option for patients who have neuroblastoma with MYCN amplification, and these findings suggest that MYCN/MYC amplification may be a predictive biomarker for BET inhibitor clinical activity. However, as with other epigenetic-based cancer therapies, a major concern lies in the inherent lack of specificity of small molecules targeting these broadly acting gene expression regulators, and the array of on-target and off-target toxicities needs to be clearly defined in the preclinical arena. Other than MYCN, focal amplifications are rare in diagnostic neuroblastomas, occurring with any significant frequency only at the ALK locus.

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The most common genomic aberration of neuroblastoma cells is somatic gain of the distal portion of chromosome 17q, which occurs in at least 50% of primary tumors, portends an overall poor prognosis, and is frequently associated with other biomarkers of aggressive disease such as MYCN amplification, older age, and chromosome 1p deletion. Remarkably, however, despite years of investigation, the mechanism by which 17q gain results in a more aggressive malignancy remains to be identified.

CHROMOSOMAL ALLELIC DELETIONS AND TUMOR-SUPPRESSOR GENES

It also has been demonstrated that allelic losses at chromosome 1p and chromosome 11q carry integral prognostic information in neuroblastoma. Loss of heterozygosity at chromosome 1p36 occurs in 23% to 35% of primary neuroblastomas and is associated with other high-risk clinical and genomic features, such as older age, MYCN amplification, and metastatic disease. Loss of chromosome 11q occurs in approximately 33% of neuroblastomas and is associated with a poor prognosis; however, it is inversely associated with MYCN amplification, making it a potential biomarker for aggressive subphenotypes in patients without MYCN amplification. However, again, despite years of tireless investigation, no gene at 1p or 11q has been identified that harbors the recurrent inactivating mutations required to fulfill the classic criteria of a bona fide tumor-suppressor gene. It is likely that the very large deletions at these (and other) loci have an impact on many genes, with aberrant methylation or other epigenetic alterations further down-regulating gene expression.

SOMATIC MUTATIONS

Pediatric malignancies in general have a much lower frequency of somatic mutation than most adult carcinomas. Accordingly, despite recent extensive efforts using next-generation sequencing techniques, few recurrent somatic mutations have been discovered in neuroblastoma tumors at the time of diagnosis (Table 2). ALK is the most commonly somatically mutated gene in neuroblastoma, and up to 14% of tumors have genetic missense alterations or gene amplification events. Putative loss-of-function genetic alterations in the RNA-helicase ATRX (α-thalassemia/mental retardation syndrome X-linked) have been identified in up to 10% of neuroblastomas; they are enriched in older patients and may help explain the more indolent phenotype observed in this population. Similar to other tumors, neuroblastomas that are deficient in ATRX appear to undergo the telomerase-independent telomere maintenance mechanism termed alternate lengthening of telomeres, which may be a marker of an indolent course with primary chemotherapy resistance. Other chromatin remodeling proteins are also significantly mutated in neuroblastoma as single nucleotide alterations or deletions of the AT-rich interactive domain 1A/1B (ARID1A/1B) genes have also been detected in a significant subset of clinically aggressive neuroblastomas. Other recurrent somatic mutations have been detected rarely in neuroblastoma tumors (≤5% of primary tumors), including genetic alterations in PTEN, odd O2/ten-m homolog 3 (ODZ3), protein tyrosine phosphatase nonreceptor type 11 (PTPN11), MYCN (P44L [a substitution of proline with leucine at codon 44]), and neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS). Clearly, efforts at improving the outcomes of patients with neuroblastoma cannot focus exclusively on developing small molecules that target mutated oncogenes, and complementary strategies must be defined.

CLONAL EVOLUTION: CURRENT UNDERSTANDING AND DRUGGABLE OPPORTUNITIES FOR RELAPSED PATIENTS

Although clonal evolution has been studied in neuroblastoma since Peter Nowell first introduced the concept in the 1970s, until very recently, neither clonal evolution nor the regional tumor heterogeneity that likely proceeds it has been comprehensively studied in neuroblastoma with next-generation sequencing efforts. More recent focused studies using genomic deep-sequencing efforts have suggested that clonal evolution also results in the acquisition of targetable somatic aberrations in known oncogenic pathways, and early evidence suggests that these converge on the mitogen-activated protein (MAP) kinase (MAPK) pathway. Although the MAPK pathway is a key driver of oncogenicity in several human malignancies, until recently, this was not believed to be the case in neuroblastoma, because MAPK pathway mutations are rare in primary neuroblastoma tumors (although NRAS was discovered in neuroblastoma). However, in a recent, comprehensive, deep-resequencing study of 23 relapsed/refractory neuroblastomas, 78% of relapsed specimens
<table>
<thead>
<tr>
<th>Clinically Actionable Pathway/Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Prevalence at Diagnosis [References]</th>
<th>Prevalence at Relapse [References]</th>
<th>Biomarker [References]</th>
<th>Therapeutic Strategy [References]</th>
<th>Level of Evidence&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALK</strong></td>
<td>Mutation/focal amplification&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8%-14% (Sausen 2013&lt;sup&gt;39&lt;/sup&gt;, Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Molenaar 2012&lt;sup&gt;31&lt;/sup&gt;, Cheung 2012&lt;sup&gt;42&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>26%-43% (Schleiermacher 2014&lt;sup&gt;58&lt;/sup&gt;, Elelveid 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>ALK mutation/amplification</td>
<td>ALK inhibition (Barone 2013&lt;sup&gt;35&lt;/sup&gt;, Carpenter &amp; Mosse 2012&lt;sup&gt;38&lt;/sup&gt;)</td>
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<tr>
<td><strong>RAS-MAPK pathway</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PTPN11</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<td></td>
<td>FGFR1</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<td></td>
<td>BRAF</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td>NRAS</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<td>KRAS</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<td></td>
<td>HRAS</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Shukla 2012&lt;sup&gt;87&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<td></td>
<td>NF1</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;)</td>
<td>9% (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>RAS-MAPK pathway mutation</td>
<td>MEK inhibition (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
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<tr>
<td><strong>Cell cycle control</strong></td>
<td>CDK4/6 amplification</td>
<td>4% (Molenaar 2012&lt;sup&gt;90&lt;/sup&gt;)</td>
<td>—</td>
<td>MYCN amplification (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
<td>CDK4/6 inhibition (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
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<td></td>
<td>Cyclin D1 amplification</td>
<td>2%-15% (Molenaar 2003&lt;sup&gt;93&lt;/sup&gt;, 2012&lt;sup&gt;90&lt;/sup&gt;)</td>
<td>—</td>
<td>MYCN amplification (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
<td>CDK4/6 inhibition (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
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<td></td>
<td>CDKN2A deletion</td>
<td>0%-20% (Molenaar 2012&lt;sup&gt;90&lt;/sup&gt;, Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;, Omura-Minamisawa 2001&lt;sup&gt;95&lt;/sup&gt;)</td>
<td>13%-22% (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;, Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;)</td>
<td>MYCN amplification (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
<td>CDK4/6 inhibition (Rader 2013&lt;sup&gt;35&lt;/sup&gt;, Gogolin 2013&lt;sup&gt;91&lt;/sup&gt;)</td>
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<td><strong>DNA damage pathway</strong></td>
<td>TP53</td>
<td>1%-8% (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;, Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;, Omura-Minamisawa 2001&lt;sup&gt;95&lt;/sup&gt;)</td>
<td>15% (Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;)</td>
<td>TP53 mutation</td>
<td>Unknown</td>
</tr>
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<td></td>
<td>MDM2 amplification</td>
<td>13% (Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;)</td>
<td>13% (Carr-Wilkinson 2010&lt;sup&gt;94&lt;/sup&gt;)</td>
<td>MDM2 mutation</td>
<td>MDM2 inhibition (Chen 2015&lt;sup&gt;36&lt;/sup&gt;, Gambel 2012&lt;sup&gt;97&lt;/sup&gt;, Van Maerken 2011&lt;sup&gt;98&lt;/sup&gt;)</td>
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<tr>
<td><strong>Transcriptional control</strong></td>
<td>MYCN amplification</td>
<td>16%-38% (Brodeur 1984&lt;sup&gt;10&lt;/sup&gt;, Seeger 1985&lt;sup&gt;11&lt;/sup&gt;, Cohn 2009&lt;sup&gt;17&lt;/sup&gt;)</td>
<td>—</td>
<td>MYCN amplification</td>
<td>BET inhibition (Delmore 2011&lt;sup&gt;81&lt;/sup&gt;, Puisant 2013&lt;sup&gt;83&lt;/sup&gt;)</td>
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<td></td>
<td>MYCN mutation</td>
<td>Rare (Pugh 2013&lt;sup&gt;40&lt;/sup&gt;)</td>
<td>—</td>
<td>MYCN mutation</td>
<td>BET inhibition (Delmore 2011&lt;sup&gt;81&lt;/sup&gt;, Puisant 2013&lt;sup&gt;83&lt;/sup&gt;)</td>
</tr>
<tr>
<td><strong>Chromatin modification</strong></td>
<td>ATRX deletion/mutation</td>
<td>9%-22% (Pugh 2013&lt;sup&gt;10&lt;/sup&gt;, Cheung 2012&lt;sup&gt;42&lt;/sup&gt;)</td>
<td>17% (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>ATRX deletion/mutation</td>
<td>Unknown</td>
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<tr>
<td></td>
<td>ARID1A/ARID1B deletion/mutation</td>
<td>11% (Sausen 2013&lt;sup&gt;39&lt;/sup&gt;)</td>
<td>Rare (Eleveld 2015&lt;sup&gt;89&lt;/sup&gt;)</td>
<td>ARID1A/B deletion/mutation</td>
<td>Unknown</td>
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Abbreviations: ALK, anaplastic lymphoma kinase; ARID1A/ARID1B, AT rich interactive domain 1A and 1B; ATRX, α-thalassemia/mental retardation syndrome X-linked; BET, bromodomain and extra-terminal; BRAF, B-Raf proto-oncogene, serine/threonine kinase; CDK4/6, cyclin-dependent kinases 4 and 6; CDKN3A, cyclin-dependent kinase inhibitor 2A; FGFR1, fibroblast growth factor receptor 1; HRAS, Harvey rat sarcoma viral oncogene homolog; KRAS, Kirsten rat sarcoma viral oncogene homolog; MDM2, MDM2 proto-oncogene, E3 ubiquitin protein ligase; MEK, Mitogen-activated protein/extracellular signal-regulated kinase kinase; MYCN, v-myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog; NF1, neurofibromin 1; NRAS, neuroblastoma RAS viral (v-ras) oncogene homolog; PTPN11, protein tyrosine phosphatase nonreceptor type 11; RAS-MAPK, rat sarcoma oncogene/mitogen activated protein kinase; TP53, tumor protein 53.

<sup>a</sup>Clinically actionable somatic mutations in neuroblastoma tumors at the time of diagnosis are rare. Clonally derived driver alterations in targetable oncogenic pathways occur more commonly in the relapsed neuroblastoma genome. Many of these potentially clinically actionable genes/pathways have identifiable biomarkers and potential therapeutic strategies with varying degrees of supporting evidence as indicated.

<sup>b</sup>For levels of evidence, 1 indicates clinical evidence of efficacy in neuroblastoma; 2, extensive preclinical studies with efficacy in neuroblastoma; 3, preliminary preclinical mechanistic studies in neuroblastoma/other malignancy.

<sup>c</sup>Taken together, activating RAS-MAPK pathway aberrations occur in approximately 80% of relapsed neuroblastomas (Eleveld 2015<sup>89</sup>).

<sup>d</sup>Rare indicates <5% prevalence.
had a clonally enriched somatic mutation identified that was predicted to activate the MAPK pathway, including not only ALK aberrations but also somatic aberrations in NRAS; Kirsten RAS viral oncogene homolog (KRAS); Harvey RAS viral oncogene homolog (HRAS); B-Raf proto-oncogene, serine/threonine kinase (BRAF); PTPN11; fibroblast growth factor receptor 1 (FGFR1); and neurofibromin 1 (NFI). Furthermore, more than 60% of human-derived neuroblastoma cell lines, the majority of which are established at disease relapse, similarly carry a MAPK pathway-activating genomic alteration.89

These findings highlight a potential therapeutic strategy in the relapsed setting, for example, with Mitogen-activated protein/extracellular signal-regulated kinase kinase 1 (MEK1) and MEK2 inhibitors (Table 2). Also worth noting is the recent observation that MAPK pathway inhibition was able to reverse retinoid resistance in neuroblastoma cells, because the tumor-suppressor NFI was identified as a key modulator of neuroblastoma cell retinoid sensitivity, an effect that could be reversed with MEK inhibition.90 Although it has been known for decades that retinoids can induce terminal differentiation of neuroblastoma cells in vitro, and the use of isotretinoin along with antiganglioside GD2-targeted immunotherapy has become the standard of neuroblastoma maintenance therapy, the response to retinoids is heterogeneous, and retinoids fail to prevent disease relapse in a substantial proportion of patients with neuroblastoma.7,8,110-112

Finally, concerning MAPK pathway germline aberrations, neuroblastoma rarely can arise in the setting of certain genetic syndromes, such as Noonan,51,52 Costello,51 and neurofibromatosis type 1,50 which are associated with PTPN11, HRAS, and NFI germline mutations, respectively (Fig. 1). Taken together, these data support a strategy targeting the MAPK pathway, at least in relapsed/refractory neuroblastoma. Inhibiting this pathway may ultimately have the dual effect of down-regulating a clonally acquired oncogenic driver pathway in relapsed neuroblastomas as well as enabling the terminal differentiation of these tumors.

The cyclin D1 (CCND1)/cyclin-dependent kinase 4 (CDK4)/CDK6/retinoblastoma 1 (RB1) cell cycle regulatory pathway is also a significant contributor to neuroblastoma pathogenesis, and the activity of this pathway correlates with MYCN amplification, providing a biomarker for potentially susceptible patients.90-93,113-118 Although somatic activating mutations in this pathway are rare at diagnosis in neuroblastoma, several genetic aberrations have been identified that increase CDK4/CDK6 expression and kinase activity and, ultimately, drive cell cycle progression, including genomic amplification of CCND1 and CDK4 or deletion of cyclin-dependent kinase inhibitor 2A (CDKN2A),40,90,93,114-116,118 and the frequency of these aberrations may be higher in the relapsed neuroblastoma genome (Table 2).89,94 Overall, approximately 33% of primary diagnostic neuroblastomas may contain genomic aberrations that implicate the G1 cell cycle-regulatory genes.90,113 In an RNA-interference screen of the protein kinase, CDK4 was identified as one of the key kinases that drives neuroblastoma cell proliferation in neuroblastoma preclinical models.117 Genetic depletion of CDK4 not only decreases neuroblastoma cell proliferation but also partially restores G-S cell cycle arrest and resensitizes neuroblastoma cells to doxorubicin-mediated cell death.92 Finally, dual pharmacologic inhibition of CDK4/CDK6 with a small-molecular inhibitor of CDK4/CDK6 induced cell cycle arrest and cellular senescence in an MYCN amplification-dependent manner in neuroblastoma models.91

It is unlikely that a single targeted drug will be curative in the relapsed neuroblastoma setting, and the development of acquired resistance to a single agent is a real possibility. Thus, an additional challenge is to identify rational, synergistic drug combinations based on the activating genomic parameters present in individual relapsed tumors. Clearly, a next-generation sequencing-based trial is needed in neuroblastoma to define the precise subset of acquired somatic changes in real time in the relapsed, high-risk neuroblastoma genome, thereby creating an opportunity to offer these children novel combination therapies targeting the specific pathways that are altered in their tumors (Table 2). Recently, this clinical trial idea has been proposed as the Next Generation Personalized Neuroblastoma Therapy (NEPENTHE) trial, a phase 1B/2 clinical trial for patients with relapsed/refractory neuroblastoma in which tumor biopsies and next-generation sequencing results will be used for novel drug combination therapy assignment that should open in late 2015. Finally, although the current focus of targeted therapy research is directed toward the relapsed, high-risk neuroblastoma genome and the activated pathways in this clinical setting, as this landscape becomes more clearly defined, it will also be important to consider and study the role of the up-front targeting of these driver pathways in high-risk neuroblastoma treatment.

DISEASE MONITORING AND CIRCULATING TUMOR DNA IN NEOBLASTOMA
Polymerase chain reaction-based and immunocytology-based methods targeting significantly overexpressed genes in neuroblastoma have been studied and verified as
sensitive markers of minimal residual disease.\textsuperscript{119} However, these methods are reliant on gene expression and are unable to capture genomic alterations, including those identified at diagnosis and those that are clonally acquired and may be drivers in the relapsed neuroblastoma tumor. Historically, pediatric oncologists have been reluctant to prescribe an invasive procedure in a patient with relapsed, high-risk neuroblastoma, especially given the diagnostic sensitivity and specificity of \textsuperscript{131}I-metaiodobenzylguanidine scintigraphy. Furthermore, biopsy of relapsed tumors has additional challenges, including their often difficult locations (eg, paraspinal or within bone) and these tumors often demonstrate significant morphologic heterogeneity, including large regions of tumor necrosis from prior treatment. In addition, neuroblastoma relapses often occur at multiple anatomic sites; and, because regional and metastatic tumor heterogeneity studies of other cancers have indicated that distinct driver aberrations can be unique to a metastatic site, a biopsy of 1 site of neuroblastoma relapse may not comprehensively identify all acquired mutations.\textsuperscript{120} Consequently, acquired oncogenic vulnerabilities in the relapsed, high-risk neuroblastoma tumor may go unrecognized if tumor genomic profiling is reliant on a single biopsy or on traditional minimal residual disease approaches, and a more effective means of comprehensively and noninvasively sampling tumor cells in this setting is needed. The assessment of circulating tumor DNA (ctDNA) using a \textit{liquid biopsy} may directly address and potentially obviate many of these concerns. Methods for detecting ctDNA have evolved, and techniques now exist that can detect rare ctDNA variants in a complex mixture of DNA with high sensitivity, and these techniques have the ability to detect the tumor-derived DNA variants (ie, point mutations) that make up \( \leq 0.01\% \) of the total cell-free DNA in the plasma.\textsuperscript{121,122} It has been observed that children with neuroblastoma have high levels of ctDNA, especially at the time of diagnosis and relapse, and tumor-specific genomic alterations have reliably been detected in sera from these children, including \textit{MYCN} amplification, gain of chromosome 17q, and (most recently) \textit{ALK} mutations using a droplet digital polymerase chain reaction system.\textsuperscript{122-126} Although potentially useful for monitoring disease burden and response to treatment, the real therapeutic potential of detecting ctDNA may be in the setting of relapsed neuroblastoma, in which the acquisition of actionable somatic aberrations may have been acquired through clonal evolution and may represent a therapeutic opportunity to initiate targeted therapy in these patients, as discussed above. Finally, the use of serial ctDNA detection for prognostic purposes has not been studied in neuroblastoma but may also offer an opportunity to capitalize on this technology.

\section*{CONCLUSIONS AND FUTURE DIRECTIONS}

Many significant improvements in neuroblastoma diagnosis, risk stratification, and treatment have been made over the last few decades. However, because substantial numbers of children cannot be cured, and because those who are cured often suffer many debilitating, long-term side effects, it is clear that efforts still are needed to better understand the aberrantly expressed genes and activated pathways and to identify novel therapeutic targets in neuroblastoma. As next-generation genomic tools continue to advance in the coming decade, it will be imperative to integrate these complex genomic, biologic, and clinical data to best refine risk-stratification and treatment strategies. Furthermore, it will be vital to identify the driver pathways and targetable aberrations at the level of a given tumor so that they can be leveraged clinically if a small-molecule or immunotherapeutic approach exists for targeting that given alteration. The Therapeutically Applicable Research to Generate Effective Treatments (TARGET) project will continue to play a major role in using a comprehensive molecular profiling approach, including gene expression, methylation, and chromosome copy number analysis, in addition to whole-genome and transcriptome sequencing techniques to characterize both primary and relapsed neuroblastomas with the objective of discovering novel therapeutics for patients with high-risk disease (https://ocg.cancer.gov/programs/target/projects/neuroblastoma). Finally, continued work on defining the genetic basis of neuroblastoma and understanding the complex biology at GWAS loci should also identify additional key molecular targets that warrant well designed translational studies.

Patients who have relapsed, high-risk neuroblastoma remain a significant challenge for pediatric oncologists, and no curative treatment currently exists. Clonally acquired somatic alterations that occur under the selective pressure of cytotoxic chemoradiotherapy offer clinically tractable targets, and this strategy is currently being studied in a prospective trial. Clearly, however, efforts at improving neuroblastoma outcomes cannot focus exclusively on developing small molecules that target mutated or dysregulated oncogenes, and complementary strategies must be defined. Recently, the first neuroblastoma immunotherapy strategy using a chimeric monoclonal antibody targeting the distialoganglioside GD2 on neuroblastoma cells demonstrated improved survival in a randomized
phase 3 clinical trial, credentialing immunotherapy for this disease. Neuroblastoma is an ideal malignancy to target with immunotherapy, because it derives from developing neural crest cells and, thus, continues to selectively express lineage-specific cell surface markers that may not be widely present on mature, nonembryonic tissues. The focus of current efforts is to determine whether more therapeutically ideal, differentially expressed cell surface molecules are present on neuroblastoma cells and to determine whether the targeting of these molecules will be efficacious in children with high-risk neuroblastoma as well as those with other aggressive pediatric malignancies. The primary focus of a current Stand Up To Cancer-St. Baldrick’s Foundation (SU2C-SBF) cosupported “Pediatric Cancer Dream Team” is to bring the fields of genomics and immunology together to further catalyze the discovery of novel immunotherapeutic targets and deliver novel protein-based and cellular treatments across aggressive pediatric malignancies, including neuroblastoma. Finally, given the complexity of the neuroblastoma genome and the common acquisition of mutations at relapse, neontogen discovery may also serve as an important aspect of defining targets for immunotherapy. Comprehensive understanding of the genetics and genomics of neuroblastoma has led to major clinical advances over the last few decades and holds great promise for continuing to drive significant improvements in clinical care in the years to come.

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CONFLICT OF INTEREST DISCLOSURES
The authors made no disclosures.

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Beiske K, Burchill SA, Cheung JY, et al. Consensus criteria for sensitive detection of minimal neuroblastoma cells in bone marrow, blood and stem cell preparations by immunocytochemistry and QRT-PCR.


