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Neuroblastoma

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Abstract

Neuroblastoma is a heterogeneous disease; tumors can spontaneously regress or mature, or display an aggressive, therapy-resistant phenotype. Increasing evidence indicates that the biologic and molecular features of neuroblastoma significantly influence and are highly predictive of clinical behavior. Because of this, neuroblastoma has served as a paradigm for biological risk assessment and treatment assignment. Most current clinical studies of neuroblastoma base therapy and its intensity on a risk stratification that takes into account both clinical and biologic variables predictive of relapse. For example, surgery alone offers definitive therapy with excellent outcome for patients with low-risk disease, while patients at high-risk for disease relapse are treated with intensive multimodality therapy. In this review recent advances in the understanding of the molecular genetic events involved in neuroblastoma pathogenesis are discussed, and how they are impacting the current risk stratification and providing potential targets for new therapeutic approaches for children with neuroblastoma. In addition, the results of significant recent clinical trials for the treatment of neuroblastoma are reviewed.

Keywords

neuroblastoma; neuroblastic tumors; risk factors; immunotherapy; targeted therapy

Neuroblastoma is an embryonal tumor of the sympathetic nervous system, arising during fetal or early post-natal life from sympathetic cells derived from the neural crest. It is the most common solid extracranial malignancy of childhood and the most common malignant tumor in infants.[1] The overall incidence of neuroblastoma is 1 case per 100,000 children in the United States, or about 700 newly diagnosed patients per year. Neuroblastoma represents about 8% of all malignancies diagnosed in pediatric patients younger than 15 years of age but is responsible for a disproportionate percentage of pediatric cancer deaths, approximately 15%.[2] However, neuroblastoma is an extremely heterogeneous disease;[3] tumors can spontaneously regress or mature, even without therapy, or display a very aggressive, malignant phenotype that is poorly responsive to current intensive, multimodal therapy. A number of the factors responsible for this heterogeneity have been identified and increasing evidence indicates that the biologic and molecular features of neuroblastoma are highly predictive of clinical behavior. The assessment of biological risk can, therefore, be used for treatment assignment and stratification, whereby those at high risk for disease relapse are given intensive multimodal therapy, in an attempt to effect a cure. Those at low-

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risk for relapse can have treatment intensity diminished in an attempt to avoid therapy-associated toxicity, while still achieving a very high rate of cure. The predictive value of these biologic factors is important not only for the oncologist when considering appropriate chemotherapy, but also for the surgeon when considering the timing and extent of an operative procedure for a child with neuroblastoma.

Risk and treatment stratification

Prognostic variables

The most important clinical variables for children with neuroblastoma appear to be age[4] and stage[5] at diagnosis. Among the most powerful biologic factors are *MYCN* status,[6,7] histopathologic classification,[8] and DNA content (ploidy).[9,10] These variables contribute to the Children's Oncology Group (COG) risk stratification and therapeutic approach. However, these factors are currently being refined and augmented by analyses performed by the International Neuroblastoma Risk Group (INRG) Task Force. The INRG Task Force, which initially convened in 2004, is composed of investigators from the major pediatric cancer cooperative groups throughout the world. The main objective of this Task Force was to develop a consensus approach to pretreatment risk stratification of children with neuroblastoma.

Stage—International criteria for a common neuroblastoma staging system were first described in 1988, and subsequently revised in 1993.[11] The International Neuroblastoma Staging System (INSS) is a surgicopathologic staging system that depends on the completeness of resection of the primary tumor, assessment of ipsilateral and contralateral lymph nodes and the relation of a primary tumor to the midline (Table 1). Although INSS has been shown to have prognostic relevance, there have been some difficulties with its widespread use. The expertise and aggressiveness of the surgeon influence tumor stage, lymph node sampling is done erratically, and patients who are simply observed without surgery cannot be appropriately staged. Therefore, a uniform, pretreatment staging system that could be used easily throughout the world, and subject to real-time central review, was sought. Montclair et al, on behalf of the INRG proposed a new staging system in 2009 based on tumor imaging rather than the extent of surgical resection.[12] In this staging system, localized tumors are staged based on the absence (L1) or presence (L2) of one or more of 20 image defined risk factors (IDRFs). Metastatic tumors are defined as stage M. Stage MS, similar to INSS stage 4S, refers to disease with metastases limited to skin, liver and bone marrow (less than 10%) in children less than 18 months of age at diagnosis (the INSS 4S age cutoff is 12 months). These young patients can have L1 or L2 primary tumors. The IDRFs are listed in Table 2 and generally reflect encasement of vital structures, primarily vessels and nerves, as determined by diagnostic imaging studies. Absence of these factors had previously been shown to be associated with safe, complete tumor resection.[13] In a review of 661 patients in the INRG database, Montclair et al found that INRG staging had prognostic significance; patients with stage L1 disease had a significantly greater 5-year event-free survival than those with stage L2 disease (90% ± 3% vs 78% ±4%, $p = 0.001$). Although INSS is currently still the staging system used for COG patients, the INRG stage assignment is being collected prospectively on all patients for subsequent evaluation.

Age—Patient age at the time of diagnosis is another clinical variable with independent prognostic value. For all stages of disease beyond stage 1 localized tumors, patients less than one year of age have had significantly better disease-free survival rates than older children with equivalent stages of disease.[4] Subsequent data from London et al on behalf of the COG suggest that the prognostic contribution of age to outcome in patients with neuroblastoma is continuous in nature. Within clinically relevant risk stratification, they found that statistical

support exists for an age cutoff of 460 days. More recent data from Moroz et al on behalf of the INRG suggest that an age-at-diagnosis cutoff of greater than 18 months is associated with higher risk disease.[14] Current COG neuroblastoma protocols have lowered the risk classification from high to intermediate and, therefore, intensity of therapy, for patients 12–18 months of age with stage 3 disease in which the tumor has unfavorable histology, as long as it is not *MYCN* amplified, and stage 4 disease when the tumor has all favorable biologic characteristics (see below).

Histopathology—In 1984 Shimada and colleagues[15] developed an age-linked classification system of neuroblastic tumors based on tumor morphology. This was redefined by the International Neuroblastoma Pathology Classification (INPC) which was established in 1999[16] and revised in 2003.[17] Neuroblastic tumors were divided into four histologic subtypes based on the degree of surrounding Schwannian stroma: neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma-intermixed (Schwannian stroma-rich), ganglioneuroblastoma-nodular (composite, Schwannian stroma-rich/stroma-dominant and stroma-poor), and ganglioneuroma (Schwannian stroma-dominant). These tumors are assigned to one of two prognostic subgroups, favorable and unfavorable, based on histologic characteristics, including the degree of neuroblast differentiation; the nuclear morphology of neuroblastic cells (mitosis-karyorrhexis index [MKI]); and the patient's age.[18] Ganglioneuroblastoma-nodular tumors are classified based on the characteristics of the usually grossly apparent nodules (additionally, *MYCN* determination should be performed specifically on the nodule); ganglioneuroblastoma-intermixed and ganglioneuroma are always of favorable histology. In fact, ganglioneuroma is considered a benign neuroblastic tumor. Despite this, ganglioneuromas can be quite large and infiltrative, and attempts at removal associated with significant complications. In addition, survival does not seem to be influenced by extent of resection.[19] Therefore, aggressive attempts at surgical resection are not recommended. The INPC classification system became widely accepted and has proven to be useful as an independent predictor of disease outcome. The importance of this histopathologic classification was confirmed in a large, retrospective analysis reported by Shimada et al.[8] Analysis of the prognostic importance of histopathology has been confounded by the inclusion of age, itself an independent prognostic factor, in the classification. Therefore, in the INRG classification schema tumor differentiation and MKI are separated for risk stratification (see below).

Molecular abnormalities

Amplification of the MYCN proto-oncogene: Early studies of neuroblastoma showed the frequent presence of extrachromosomal double-minute chromatin bodies (DMs) and chromosomally integrated homogeneously staining regions (HSRs) characteristic of gene amplification.[20] Since that time, it has been shown that the amplified region was derived from the distal short arm of chromosome 2 (2p24) and contained the *MYCN* proto-oncogene. Overall, approximately 25% of primary neuroblastomas have *MYCN* amplification; *MYCN* amplification is present in 40% of patients with advanced disease and 5% to 10% of patients with low-stage disease.[6] Amplification of *MYCN*, defined as greater than 10 copies of the gene per cell, is associated with advanced stages of disease, rapid tumor progression, and poor outcome; therefore, it is a powerful prognostic indicator of biologically aggressive tumor behavior and has remained so since its identification in 1983.[6,7]

DNA content (ploidy): Normal human cells contain two copies of each of 23 chromosomes; thus, a normal diploid cell has 46 chromosomes. The majority (55%) of primary neuroblastomas are triploid or "near-triploid," containing between 58 and 80 chromosomes; the remainder (45%) are either "near-diploid" (35–57 chromosomes) or "near-tetraploid" (81–103 chromosomes).[21] The DNA index is the ratio of the number of chromosomes to the

expected number (i.e. 46). Therefore, diploid cells have a DNA index of 1.0, whereas near-triploid cells have a DNA index ranging from 1.26 to 1.76. Patients with near-triploid (also termed "hyperdiploid") tumors typically have favorable clinical and biologic prognostic factors and excellent survival rates, as compared with those patients who have near-diploid or near-tetraploid tumors.[10] This association was initially felt to be most important for infants with advanced disease.[22] Currently, ploidy only potentially impacts the risk group assessment of infants age 12–18 months with metastatic disease and infants with 4S disease in the COG risk stratification schema (see below).

Allelic deletions on chromosomes 1p and 11q: Deletions of genetic material in tumors suggest the presence (and subsequent loss) of a tumor suppressor gene. Early karyotype analyses of neuroblastoma-derived cell lines showed frequent deletions of the short arm of chromosome 1 (1p).[23] 1p deletions, as determined by FISH, are now felt to occur in about 30% of neuroblastomas with the smallest common region of loss located within region 1p36.[24] About 70% of advanced-stage neuroblastomas have 1p deletions,[25] an occurrence that correlates with both *MYCN* amplification and other high-risk features.[24] A recent study has demonstrated that 1p deletions are independently associated with a worse outcome in patients with neuroblastoma.[26] Although no individual tumor suppressor gene has been confirmed on chromosome 1p, recent data have identified *CHD5* as the strongest candidate tumor suppressor gene that is deleted from 1p36.31 in neuroblastoma.[27]

Deletion of the long arm of chromosome 11 (11q) also appears to be common in neuroblastoma, being present in about 40% of cases. Unbalanced deletion of 11q (loss with either retention or gain of 11p material) is inversely related to *MYCN* amplification[26,28] yet is strongly associated with other high-risk features. Recently, Attiyeh et al, on behalf of the COG, showed in a large cohort of patients that unbalanced deletion of 11q and 1p36 were independently associated with a worse outcome in patients with neuroblastoma.[29] Therefore, the duration of treatment for children with intermediate risk neuroblastoma on the current COG study is based, in part, on the 1p and 11q allelic status of the tumor.

Current COG risk stratification

On the basis of these clinical and biological prognostic variables, infants and children with neuroblastoma are currently categorized into three risk groups predictive of relapse; low, intermediate, and high-risk (Table 3). The probability of prolonged disease-free survival for patients in each group is 95% to 100%, 85% to 90%, and less than 30%.

Low-risk disease

Characteristics: This classification includes all patients with stage 1 disease or patients with stage 2A/2B disease that is not *MYCN*-amplified and where the tumor has undergone a greater than 50% resection. Also included in the low-risk group are infants with stage 4S disease that is of favorable histology, without *MYCN* amplification, and has a DNA index greater than 1.

Results: A group-wide study (COG P9641: Primary Surgical Therapy for Biologically Defined Low-Risk Neuroblastoma) was conducted from 1998–2006 to evaluate primary surgical therapy for biologically defined low-risk neuroblastoma. The overall strategy was to treat patients with low-risk neuroblastoma with surgery and supportive care only; adjuvant therapy was given only when less than 50% of the tumor was resected or when symptoms that were life- or organ-threatening developed. A probability of 3-year survival more than 95% was predicted for these patients with low-risk disease. The final results from this study have yet to be published, but it appears likely that greater than 50% resection of low-risk,

INSS stage 2A/B tumors in asymptomatic patients is sufficient therapy; adjuvant chemotherapy can be reserved for patients with disease progression or recurrence.

Current treatment: The treatment for patients with low-risk disease remains surgical resection alone, even in the presence of microscopic residual disease (stage 1), gross residual disease (stage 2A), or gross residual disease with ipsilateral lymph node involvement (stage 2B), if the tumor does not have *MYCN* amplification. Infants with stage 4S disease who are not experiencing substantial symptoms may undergo an initial biopsy and observation only, if the tumor has favorable biologic factors. It is also likely that close biochemical and sonographic observation alone can be used for the management of infants with small adrenal masses, with surgical resection being reserved for those rare cases in which there is evidence of continued growth. This hypothesis has been tested in the recently closed COG trial, ANBLOOP2: Perinatal Neuroblastoma: Expectant Observation. To be eligible, infants with an adrenal mass had to be < 6 months of age when the mass was first identified; the mass had to be < 16 ml in volume, if solid, or < 65 ml if at least 25% cystic; and disease must have been limited to the adrenal gland. The results of this study should be available soon.

Intermediate-risk disease

Characteristics: This classification includes patients age 0–12 years with stage 2A/2B disease that is not *MYCN*-amplified and where the tumor has undergone less than 50% resection (or biopsy only), patients age 0–1½ years with stage 3 disease whose tumors are not *MYCN*-amplified, patients age 1½–12 years with stage 3 disease whose tumors are not *MYCN*-amplified and are of favorable histology, infants with stage 4 disease whose tumors are not *MYCN*-amplified, and patients age 1–1½ years with stage 4 disease whose tumors are not *MYCN*-amplified, have favorable histology and DI>1. Also included in this group are infants with 4S disease who are symptomatic from their tumor and the tumor biologic characteristics are either of unfavorable histology or DI=1, or if no tissue was obtained at presentation for evaluation.

Results: A group-wide study (COG A3961: Treatment for Infants and Children with Intermediate-Risk Neuroblastoma) was conducted from 1998–2006 to further refine therapy for patients with intermediate-risk disease. The overriding aim of this study was to maintain or improve survival while minimizing both acute and long-term morbidity in this group of patients. Patients received four of the most active agents in neuroblastoma: cyclophosphamide, doxorubicin, carboplatin, and etoposide, given for either four cycles (favorable biology) or eight cycles (unfavorable biology); cycles were given every 3 weeks. Radiation therapy was not given unless there was progressive disease or an unresectable primary tumor with unfavorable prognostic features at the end of chemotherapy. The outcome after reduced chemotherapy for intermediate-risk neuroblastoma was published recently by Baker et al, on behalf of the COG.[30] The 3-year overall survival for the entire group was 96%; survival was 98% for those with favorable biologic features and 93% for those with unfavorable features.[30]

Current treatment: The current COG protocol (ANBL0531: Response- and Biology-based Therapy for Intermediate-risk Neuroblastoma), which opened in October, 2007, was recently closed to accrual. This protocol sought to further refine the minimal therapy needed to achieve the excellent outcomes for patients with intermediate-risk neuroblastoma. As such, many patients, as defined by favorable clinical and biologic factors, received a further reduction in therapy. However, those patients in whom there is loss of heterozygosity (LOH – loss of one of two normally paired chromosomal regions) at chromosome 1p or 11q (unbalanced) were not eligible for this dose reduction, as these events have been shown to be independently associated with decreased progression-free survival in patients with low-

and intermediate-risk disease.[26] Patients again received cycles of cyclophosphamide, doxorubicin, carboplatin, and etoposide given every 3 weeks. The duration of therapy (i.e., the number of cycles), depended upon which of three intermediate-risk groups a patient was placed in, with group stratification again being based on clinical and biologic risk factors (see Table 3). For almost all intermediate- risk patients, regardless of group (except Group 4, stage 4 infants), this represented a reduction in therapy, either shortening the duration (Groups 2 and 3), or downgrading from high-risk therapy (Group 4). However, as previously mentioned, patients in Groups 2 or 3 whose tumor contains chromosomal 1p LOH or unbalanced 11q LOH (or if the data were missing) were upgraded one group (i.e. received double the number of chemotherapy cycles, just as they would have on the prior intermediate-risk COG trial A3961).

The overall surgical goal in intermediate-risk patients is to perform the most complete tumor resection possible, consistent with preservation of full organ and neurologic function. This may necessitate leaving residual disease adherent to critical anatomic structures. If a primary tumor is judged by the surgeon to be unresectable, a diagnostic biopsy is generally obtained and chemotherapy initiated. Delayed surgery is performed after the prescribed number of cycles, as dictated by the Group assignment. A reduction in surgical therapy is being evaluated for infants with 4S disease as it is no longer required that they undergo resection of their primary tumor. In addition, if they are too unstable at presentation, it is no longer required that they undergo an initial biopsy in order to be eligible for enrollment on ANBL0531.

Radiation was administered only to symptomatic intermediate-risk patients when there was a risk of organ impairment due to tumor bulk not responding to initial chemotherapy, particularly in infants with 4S disease and respiratory insufficiency and patients with epidural disease and symptoms of spinal cord compression.

High-risk Disease

Characteristics: This classification includes all patients older than 1½ years with stage 4 disease, patients of any age and any stage (except stage 1) with *MYCN* amplified tumors,, patients older than 1½ years with stage 3 tumors that have unfavorable histology, and patients 1–1½ years of age with stage 4 disease that has either unfavorable histology or DI=1.

Results: The general approach to treating patients with high-risk neuroblastoma has included intensive induction chemotherapy, myeloablative consolidation therapy with stem cell rescue, and targeted therapy for minimal residual disease. Stem cell harvest is typically performed after the first two cycles of induction therapy, and resection of the primary tumor and bulky metastatic sites is attempted after the fifth cycle. The CCG-3891 protocol enrolled patients with high-risk neuroblastoma between 1991 and 1996 and was designed to assess whether myeloablative therapy in conjunction with autologous BMT improved EFS, compared with chemotherapy alone, and whether subsequent treatment with 13-*cis*-retinoic acid, a differentiating agent, would further improve EFS.[31] The results from this double-randomization study demonstrated that the 3-year EFS was significantly better in patients who underwent BMT during the first randomization (34%) than in those who did not (22%; P=0.034); in the second randomization, those who received 13-*cis*-retinoic acid after BMT experienced a significantly better 3-year EFS (46%) than those who did not receive the retinoid (29%, P=0.027). Unfortunately, the long-term survival advantage for these patients is becoming less apparent. Nevertheless, autologous stem cell transplantation and 13-*cis*-retinoic acid are now part of most current high-risk neuroblastoma protocols.

The first cooperative group high-risk neuroblastoma protocol (A3973: A Randomized Study of Purged vs. Unpurged Peripheral Blood Stem Cell Transplant Following Dose Intensive Induction Therapy for High-Risk Neuroblastoma) opened in February, 2001. Eligible patients were randomized to receive either unpurged or purged autologous stem cells. The rationale for this randomization was that although there has been some evidence that tumor cells contaminating re-infused autologous stem cells contribute to tumor relapse in patients with neuroblastoma, it is uncertain whether the removal of the tumor cells by purging the stem cell product would influence relapse rates.[32,33] This protocol was closed to accrual early (March, 2006), however, because an interim analysis showed no difference in outcome for children who received either purged or unpurged stem cell product.

Current therapy: The current COG high-risk neuroblastoma protocol, ANB0532: Phase III Randomized Trial of Single vs. Tandem Myeloablative as Consolidation Therapy for High-Risk Neuroblastoma, which opened in November, 2007, has as its primary goal, to test whether further intensification of myeloablative therapy will improve the cure rate. Randomization to either one myeloablative consolidation with a carboplatin/etoposide/melphalan preparative regimen or two myeloablative consolidations, in which the initial regimen includes thiotepa and cyclophosphamide, will occur at the completion of induction chemotherapy.

Another primary aim of this study is to determine whether additional radiation therapy delivered to gross residual disease improves local control. Four to 6 weeks after stem cell transplantation, radiation therapy is administered to the region of the primary tumor site, including involved adjacent lymph nodes. The target volume is the area of residual disease, which is determined radiographically, after induction chemotherapy but prior to delayed surgical resection, with an additional 1.5-cm margin added, even if a complete resection was ultimately achieved. Patients whose primary site has achieved a complete response at the end of induction therapy, will receive 21.6Gy to the site of primary locoregional disease while areas with gross residual disease will be treated with an additional boost of 14.4Gy (36Gy total). Sites of persistent active metastatic disease prior to stem cell transplantation (i.e., positive sites on MIBG scan or those that do not show diminished enhancement on serial bone scans) are irradiated at the same time and with the same dose as the primary site.

The role of surgery in the management of children with high-risk neuroblastoma is controversial. Several reports have suggested that patients with INSS stage 3 or 4 disease who undergo gross total resection of their primary tumor and locoregional disease experience improved local tumor control and increased overall survival;[34,35] however, other reports have had different conclusions.[36,37] The role of debulking is also unclear. However, resection of as much gross tumor as possible in patients who receive a stem cell transplant in combination with high-dose chemotherapy and total body irradiation (TBI) may be of some benefit.[38] Despite the uncertainty of the role of surgery, the COG high-risk protocol currently recommends attempting gross total resection of the primary tumor and locoregional disease in patients with high-risk neuroblastoma. Most children undergo delayed surgery after the completion of the fifth cycle of induction chemotherapy, even though tumor volume reduction plateaus after the second or third cycle of chemotherapy.[39] Other groups are performing surgery as soon as locoregional disease appears, radiographically, to be resectable.[40] Although initial surgical resection is not often appropriate for patients with neuroblastoma, the principle of resection at the earliest feasible time should be considered.

Secondary surgical objectives embedded in the current COG high-risk protocol include: 1) To determine if resection completeness is predictive of local control rate or event-free

survival. 2) To prospectively describe the complications related to efforts at local control. 3) To describe the neurologic outcomes in patients with paraspinal primary tumors.

Proposed INRG risk stratification

As mentioned previously, in an effort to establish an international consensus on pretreatment risk stratification, the INRG Task Force developed the INRG Classification System based on an analysis of 8,800 patients treated for neuroblastoma between 1990-and 2002. They used survival tree regression analyses with event free survival (EFS) as the primary endpoint to test the prognostic significance of 13 potentially prognostic factors.[41] The analyses determined that 7 of these prognostic variables (INRG stage, age [younger/older than 18 months], histology, tumor differentiation, *MYCN* status, 11q status, and ploidy) could define 16 different pretreatment risk groups (Table 4). These groups could then be divided into four categories based on expected 5-year event free survival: very low (>85% EFS, 28.2% of patients), low (>75 to ≤85% EFS, 26.8% of patients), intermediate (≥50 to 75% EFS, 9.0% of patients) and high (<50% EFS, 36.1% of patients) risk.[41] These factors are being prospectively collected on all neuroblastoma patients with the hope that these homogenous cohorts will facilitate future comparisons of risk-based trials performed throughout the world.

New treatment strategies

Immunotherapy

Neuroblastoma cells are sensitive to antibody-dependent cell-mediated cytotoxicity, as well as to complement-dependent cytotoxicity.[42] Targeted immunotherapy using antiganglioside antibodies targeting $G_{D2,}$ the predominant antigen in neuroblastoma cells, appears to be a promising approach for the treatment of advanced neuroblastomas. In a Phase II trial, the mouse monoclonal anti-G_{D2} antibody 3F8 induced a tumor response in 40% of patients with neuroblastoma resistant to chemotherapy.[43] To decrease the immunogenicity of murine antibodies, a chimeric antibody was constructed by combining the variable regions of murine IgG_3 anti–ganglioside G_{D2} antibody 14.18 and the constant regions of human IgG_1-κ.

Because the induction of antibody-dependent cell-mediated cytotoxicity with anti–ganglioside G_{D2} antibodies is enhanced by cytokines such as GM-CSF[44] and IL-2,[45] a Phase III trial, COG ANBLOO32, was conducted to determine whether treatment with ch14.18 and cytokines (GM-CSF and IL-2) together with 13-*cis*-retinoic acid improves EFS and overall survival after autologous BMT, as compared to treatment with 13-*cis*-retinoic acid alone in patients with high risk neuroblastoma. The study was stopped early because of demonstrated efficacy; immunotherapy was superior to standard therapy (2-year EFS: 66% vs. 46%, P=0.01 and 2-year OS: 86% vs. 75%, P=0.02).[46]

MIBG Therapy

Refractory neuroblastoma has been treated with ^{131}I-MIBG, because it is readily taken-up by the tumor cells.[47] In an investigation of patients with advanced chemoresistant neuroblastomas, response rates approached 33%.[48] Studies further suggest that this treatment can be used as front-line therapy, followed by chemotherapy, without significant hematologic toxicity.[49] ^{125}I-MIBG may be an even better treatment option for neuroblastomas with micrometastases or bone marrow infiltration,[50] and is being tested for the treatment of patients with “ultra high-risk” neuroblastoma (expected survival of less than 15%). A recent study found that although the overall treatment response rate (46%) was high for all patients, older patient with neuroblastoma had a significantly higher treatment response rate and exhibited a trend toward longer post-treatment overall survival, indicating

that ^{131}I-MIBG might be an effective salvage agent for neuroblastoma in this difficult to treat patient population.[51] A randomization to receive therapeutic MIBG may be included in the next COG phase III high-risk neuroblastoma protocol.

Differentiating agents

Retinoids, vitamin A derivatives, induce morphologic differentiation of neuroblastoma.[52–55] 13-*cis*-retinoic acid (isotretinoin) is a synthetic derivative of the naturally occurring all-*trans* retinoic acid. As mentioned previously, the 3-year EFS of patients who received 13-*cis*-retinoic acid (46%) as maintenance therapy on CCG 3891 was significantly higher than that of patients who received no further therapy (29%; P=0.027) and was independent of the initial randomization to either chemotherapy or autologous BMT.[31] Because of this result, all patients on the current COG high-risk protocol receive oral 13-*cis*-retinoic acid twice daily for 2 weeks followed by 2 weeks without; this treatment is continued for 6 cycles (6 months total). Group 4 patients, age 12–18 months, being treated on the current COG intermediate-risk protocol, also receive 13-*cis*-retinoic acid. Another synthetic retinoid, fenretinide (4-HPR) is currently being tested for the use in maintenance therapy for high-risk neuroblastoma. Unlike 13-cis-RA, fenretinide does not cause phenotypic changes, but produces tumor cell apoptosis and appears to be effective against cell lines that are resistant to RA.[56] A recent COG phase I study was able to achieve systemic levels of fenretinide in patients that were active against neuroblastoma *in vitro* with minimal toxicity.[57]

Angiogenesis inhibition

Angiogenesis is the biologic process of blood vessel formation. In addition to occurring as part of several normal, physiologic processes, angiogenesis is an essential component of a number of pathologic conditions, including cancer. Compelling data suggest that inhibition of angiogenesis not only prevents tumor-associated neovascularization, but also affects tumor growth and spread. Neuroblastoma growth appears to be angiogenesis-dependent and is, therefore, likely to be susceptible to antiangiogenic therapy. Animals studies have demonstrated that neuroblastoma is susceptible to a variety of angiogenesis inhibitors, including TNP-470 (a fumagillin derivative),[58–60] VEGF-Trap,[61] a truncated soluble form of the VEGF receptor-2,[62,63] and pigment epithelium-derived factor (PEDF).[64] In addition, standard chemotherapeutic agents, when given using a low continuous dosing schedule, appear capable of treating tumors that had been previously resistant to them by destroying the neovascularity required by a progressing tumor.[65] By avoiding a “maximal tolerated dose” scheduling of these drugs, the patient can forego the recovery time required between cycles, thereby preventing recovery of the chemotherapy-sensitive endothelial cells. VEGF Trap was tested in a recent COG phase I clinical trial for children with refractory solid tumors.

New targets

ALK inhibition—Recently, activating mutations in the tyrosine kinase domain of the anaplastic lymphoma kinase (*ALK*) oncogene on the short arm of chromosome 2 (2p23) were identified as germline mutations associated with hereditary neuroblastoma.[66] These mutations can also be somatically acquired although the prevalence of *ALK* activation in sporadic neuroblastoma remains to be determined. Constitutive activation of the ALK receptor tyrosine kinase by mutation or translocation appears to contribute to the malignant phenotype of a number of cancers, including neuroblastoma, making it a potentially therapeutic target. This hypothesis is supported by the recent finding of *ALK* as a molecular target by a screen of neuroblastoma cell lines with pharmacologic antagonists.[67] The orally bioavailable small molecule inhibitor of *ALK*, PF-02341066, is currently being tested in a COG phase I/II trial of relapsed/refractory solid tumors.

Aurora A kinase inhibition—Aurora A kinase is serine/threonine kinase that is expressed in all actively dividing cells and is critical for cell cycle progression.[68] It is also overexpressed in numerous types of tumors, including neuroblastoma, in which it likely serves as an oncogene.[69] Because of its central role in mitosis, inhibition of aurora A kinase may be broadly effective as an anticancer approach. Recently, MLN8237, a selective, reversible small molecule inhibitor of aurora A kinase showed *in vivo* activity against a panel of neuroblastoma xenografts that far exceeded that observed for standard agents evaluated against the panel.[70] Based on this activity, a phase II clinical trial has been initiated in the COG using this agent to treat a variety of recurrent/refractory tumors, including neuroblastoma.

TRK inhibition—Neurotrophins and their tyrosine kinase receptors are important in the development of the sympathetic nervous system and have been implicated in the pathogenesis of neuroblastoma. Three receptor-ligand pairs have been identified: TrkA, the primary receptor for nerve growth factor (NGF); TrkB, the primary receptor of brain-derived neurotrophic factor (BDNF); and TrkC, the receptor for neurotrophin-3 (NT-3).[71] TrkA appears to mediate differentiation of developing neurons or neuroblastoma in the presence of NGF ligand, and apoptosis in the absence of NGF.[72] High TrkA expression is associated with favorable tumor biology and good outcome[73] and is inversely correlated with *MYCN* amplification.[74] Conversely, the TrkB/BDNF pathway appears to promote neuroblastoma survival through autocrine or paracrine signaling, especially in *MYCN*-amplified tumors.[75] TrkB is expressed in about 40% of neuroblastomas, usually advanced-stage disease. TrkC is expressed in approximately 25% of neuroblastomas and is strongly associated with TrkA expression.[76] Although the exact function of the Trk receptors in the pathogenesis of neuroblastoma is unknown, they remain attractive therapeutic targets. Studies are ongoing to test agonists of TrkA in an attempt to induce cellular differentiation. Conversely, blocking the BDNF/TrkB signaling pathway with Trk-specific tyrosine kinase inhibitors, such as CEP-751, may induce apoptosis by blocking crucial survival pathways.[75,77–79]

Tubulin binding agents—Microtubules play a critical role in the migration of replicated chromosomes during cell division. Tubulin binding agents can inhibit this process, thereby blocking the cell cycle in the G_2/M phase, ultimately resulting in the induction of apoptosis.[80] ABT-751 is an orally bioavailable antimitotic agent that binds β-tubulin and inhibits polymerization of microtubules.[81] Pre-clinical studies of this agent found it to be most effective in neuroblastoma models and a phase I study showed it to be safe.[82,83] The results of a recently concluded phase II trial of ABT-751 in children with relapsed or refractory neuroblastoma are pending publication.

Epigenetic targeting

The hallmark of cancer is dysregulated gene expression. However, not only do genetic factors influence gene expression but epigenetic factors do as well, with these factors being at least as important as genetic changes in their contribution to the pathogenesis of cancer. Epigenetic alterations are defined as those heritable changes in gene expression that do not result from direct changes in DNA sequence. Mechanisms of epigenetic regulation most commonly include DNA methylation, modification of histones, and changes in microRNA (miRNA) expression.

DNA methylation—DNA methylation is a reversible process that involves methylation of the fifth position of cytosine within CpG dinucleotides present in DNA. These dinucleotides are usually in the promoter regions of genes; methylation of these sites typically causes gene silencing, thereby preventing expression of the encoded proteins. This process is part of the normal mechanism for imprinting, X-chromosome inactivation and generally keeping large

areas of genomic DNA silent, but may also contribute to the pathogenesis of cancer by silencing tumor suppressor genes. Genome-wide DNA methylation analysis of neuroblastic tumors revealed that hypermethylation events are extensive and contribute to the clinicopathologic features of these tumors.[84] Promoter methylation resulting in silencing of caspase 8, a protein involved in apoptosis, for example, likely contributes to the pathogenesis of *MYCN*-amplified neuroblastoma.[85] Brief exposure of caspase 8-deficient neuroblastoma cells to low levels of demethylating agents results in the reexpression of caspase 8 and the resensitization of the cells to chemotherapeutic drug-induced apoptosis. A phase I clinical trial was conducted through COG in which decitabine (5-aza-2′-deoxycytidine), an agent known to interfere with DNA methylation, was given, together with doxorubicin and cyclophosphamide, to children with relapsed/refractory solid tumors. Unfortunately, doses of decitabine capable of producing clinically relevant biologic effects were not well tolerated with this combination.[86]

Histone modification—Histones are the proteins that give structure to DNA, and together with the DNA form the major components of chromatin. Alterations in histones can mediate changes in chromatin structure. The compacted form of DNA, termed heterochromatin, is largely inaccessible to transcription factors and, therefore, genes in the affected regions are silent. Other modifications of histones can cause DNA to take a more open or extended configuration (euchromatin), allowing for gene transcription. Histones can be modified by a number of different processes including methylation and acetylation, mediated by histone acetyl transferases (HAT) and deacetylases (HDAC), and histone methyltransferases (HMT). Each of these processes alters histone function, which, in turn alters the structure of chromatin and, therefore, the accessibility of DNA to transcription factors. Vorinistat (SAHA) is a histone deacetylase inhibitor (HDACi) that impairs the ability of HDACs to repress gene transcription, thereby effecting cell cycle arrest and/or apoptosis. This drug has recently been tested, in combination with bortezomib, a proteasome inhibitor, in a recent phase I COG trial. A recent pre-clinical study suggests that vorinistat can also function as a radiosensitizer in neuroblastoma.[87]

Micro RNA—miRNAs are a group of small, noncoding RNAs that appear to function in gene regulation. These miRNAs are single–stranded RNA fragments of 21–23 nucleotides that are complementary to encoding mRNAs.[88] Their function is to down-regulate expression of target mRNAs; it is estimated that miRNAs regulate the expression of about 30% of all human genes.[89] miRNAs are involved in a number of fundamental biologic processes, including development, differentiation, cell cycle regulation and senescence. However, broad analyses of miRNA expression levels has demonstrated that many miRNAs are dysregulated in a variety of different cancer types, including neuroblastoma and other pediatric tumors,[90] frequently losing their function as gene silencers/tumor suppressors. The activity of miRNAs, like gene expression, is also under epigenetic regulation. Therapeutic targeting of miRNA in neuroblastoma is currently being explored.[91]

Other recent molecular advances

Recently, micro-array technologies have generated extensive amounts of data that have aided in identifying genomic (DNA) and transcriptomic (RNA) abnormalities associated with neuroblastoma. In addition, these abnormalities have been shown to have significant predictive power when anticipating outcome for these patients.[92,93] Many of these findings were generated by large scale genome-wide association studies (GWAS). This is a technique whereby all or most of the genes of patients with neuroblastoma are analyzed to find differences with the population as a whole, looking for variations that are associated with the development and aggressiveness of neuroblastoma.

One such type of variation is a single-nucleotide polymorphism (SNP) in which there is a variation in the DNA sequence for a single nucleotide between children with neuroblastoma and those without, and with varying degrees of tumor phenotype. Another type of variation is copy-number variation (CNV) which is an alteration of the DNA resulting in an abnormal number of copies of one or more sections of the DNA. CNVs correspond to relatively large regions of the genome that have been deleted or duplicated.

Copy number variations

One method for detecting CNVs is by comparative genomic hybridization (CGH). Early CGH studies showed that gain of genetic material on the long arm of chromosome 17 (17q) is perhaps the most common genetic abnormality in neuroblastomas, occurring in approximately 75% of primary tumors.[94] It is unclear, however, at this time, how extra copies of 17q contribute to the malignant phenotype of neuroblastoma and which gene(s) on 17q are the critical ones. Candidate genes include *survivin* and *PPM1D*.[95,96] Nevertheless, gain of chromosome 17q is strongly associated with other known prognostic factors, but it may also be a powerful predictor of adverse outcome.[97] More recently, GWAS studies have shown that inherited copy number variation at chromosome 1q21.1 is associated with neuroblastoma, implicating a neuroblastoma breakpoint family gene in early neuroblastoma genesis.[98]

Single nucleotide polymorphisms

Other molecular studies have revealed that common genetic variation at chromosome bands 6p22[99] and 2q35[100] are associated with susceptibility to, and likely contribute to the etiology of, high-risk neuroblastoma, providing the first evidence that childhood cancers also arise owing to complex interactions of polymorphic variants. More recently a GWAS study has identified common polymorphisms including germline single nucleotide polymorphism risk alleles and somatic copy number gain, resulting in increased expression of the cysteine-rich transcriptional regulator LIM domain only 1 (LMO1) at 11p15.4. These have been shown to be strongly associated with susceptibility to developing neuroblastoma, and often are associated with advanced disease and poor survival.[101]

Mutations

Linkage studies showed an association of 2p23-p24 abnormalities in patients with familial neuroblastoma which eventually led to the identification of *ALK* mutations on 2p23.1 inherited as the germ line abnormality associated with familial neuroblastoma. Further studies have identified loss of function mutations in the homeobox gene *PHOXB2* on 4p13 that are also associated with familial neuroblastoma, particularly when occurring together with Hirschsprung's disease and/or central hypoventilation.[102]

Efflux proteins

Multidrug transporter genes encode proteins that serve as drug efflux pumps whose expression in neuroblastoma appears to be correlated with *MYCN* amplification and poor prognosis.[103,104] The presence of these multidrug resistance genes that encode the ATP-binding cassette (ABC) superfamily may explain why neuroblastomas initially respond well to chemotherapy but subsequently become resistant. Recent data suggest that ABC transporters may also modulate neuroblastoma behavior independent of their effect on chemotherapy efflux.[105]

Conclusions

As more information regarding diagnostically and prognostically useful genetic markers of neuroblastoma become available, therapeutic strategies will change accordingly. In addition, molecular profiling will lead to new drug development designed to induce differentiation of tumor cells, block dysregulated growth pathways, or reactivate silenced apoptotic pathways. One of the most exciting prospects for improving the therapeutic index, as well as overcoming the problem of tumor resistance to therapy, involves targeted therapy. These new agents can be used in concert with traditional regimens; some may be used independently.

Many of these complex studies are being conducted among collaborating and cooperating groups. Genetic analyses are being performed through the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program in the National Cancer Institute. Pre-clinical studies are being performed to test new drugs and drug combinations at high throughput against neuroblastoma cells *in vitro* and xenografts *in vivo* in the Pediatric Preclinical Testing Program (PPTP). Finally, large clinical trials continue to be conducted by the COG. In addition, several universities and children's hospitals have formed a consortium funded by the National Cancer Institute to test promising new therapies for neuroblastoma. The New Approaches to Neuroblastoma Therapy (NANT) consortium was formed to organize closely collaborating investigators whose laboratory programs are developing novel therapies for high-risk neuroblastoma. Those with promising results will be considered for more extensive national testing. Finally, results from clinical trials world-wide can now be compared using standard INRG risk stratification factors.

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TABLE 1

International Neuroblastoma Staging System

1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with primary tumor may be positive).
2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically.
2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically.
3	Unresectable unilateral tumor infiltrating across the midline,* with or without regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement; or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement.
4	Any primary tumor with dissemination to distant lymph nodes, bone marrow, bone, liver, skin and/or other organs (except as defined for stage 4S)
4S	Localized primary tumor (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver and/or bone marrow† (limited to infants <1 year of age).

*The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.

†Marrow involvement in stage 4S should be minimal, that is, <10% of total nucleated cells identified as malignant on bone marrow biopsy or on marrow aspirate. More extensive marrow involvement would be considered to be stage

4. The MIBG scan (if performed) should be negative in the marrow.

(Adapted from Brodeur GM, Pritchard J, Berthod F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging and response to treatment. J Clin Oncol 11:1446, 1999)

TABLE 2

Objective Surgical Risk Factors for primary resection of localized neuroblastoma

1	**Neck:**
	1. Tumor encasing major vessel(s) (e.g. carotid artery, vertebral artery, internal jugular vein)
	2. Tumor extending to base of skull
	3. Tumor compressing the trachea
	4. Tumor encasing the brachial plexus
2	**Thorax:**
	1. Tumor encasing major vessel(s) (eg. subclavian vessels, aorta, superior vena cava)
	2. Tumor compressing the trachea or principal bronchi
	3. Lower mediastinal tumor, infiltrating the costo-vertebral junction between T9 and T12 (may involve the artery of Adamkiewicz perfusing the lower spinal cord)
3	**Abdomen:**
	1. Tumor infiltrating the porta hepatis and/or the hepatoduodenal ligament
	2. Tumor encasing the origin of the celiac axis, and/or the superior mesenteric artery
	3. Tumor invading one or both renal pedicles
	4. Tumor encasing the aorta and/or vena cava
	5. Tumor encasing the iliac vessels
	6. Pelvic tumor crossing the sciatic notch
4	**Dumbbell tumors with symptoms of spinal cord compression:** Any location
5	**Infiltration of adjacent organs/structures:** Diaphragm, kidney, liver, duodeno-pancreatic block, and mesentery

(Adapted from Cecchetto G, Mosseri V, DeBernardi B. et al. Surgical risk factors in primary surgery for localized neuroblastoma: the LNESG1 study of the European International Society of Pediatric Oncology Neuroblastoma Group. J Clin Oncol 2005: 23:8483-9)

TABLE 3

Children's Oncology Group risk stratification for children with neuroblastoma

Risk Stratification	INSS Stage	Age	Biology
Low			
Group 1			
	1	Any	Any
	2A/2B (>50% resected)	Any	*MYCN*-NA, any histology/ploidy
	4S	<365 days	*MYCN*-NA, FH, DI>1
Intermediate			
Group 2			
	2A/2B (<50% resected or Bx only)	0–12 years	*MYCN*-NA, any histology/ploidy*
	3	<365 days	*MYCN*-NA, FH, DI>1*
	3	≥365 days - 12 years	*MYCN*-NA, FH*
	4S (symptomatic)	<365 days	*MYCN*-NA, FH, DI>1*
Group 3			
	3	<365 days	*MYCN*-NA, either UH or DI=1*
	4	<365 days	*MYCN*-NA, FH, DI>1*
	4S	<365 days	*MYCN*-NA, either UH or DI=1*; or unknown biology
Group 4			
	4	<365 days	*MYCN*-NA, either DI=1 or UH
	3	365 -<547 days	*MYCN*-NA, UH, any ploidy
	4	365 -<547 days	*MYCN*-NA, FH, DI>1
High			
	2A/2B, 3, 4, 4S	Any	*MYCN*-amplified, any histology/ploidy
	3	≥ 547 days	*MYCN*-NA, UH, any ploidy
	4	365 - >547 days	*MYCN*-NA, UH or DI=1
	4	> 547 days	Any

*If tumor contains chromosomal 1p LOH or unb11qLOH, or if data are missing, treatment assignment is upgraded to next Group.

MYCN-NA: *MYCN* not amplified, FH: favorable histology, UH: unfavorable histology, DI: DNA index

Table 4

International Neuroblastoma Risk Group (INRG) pretreatment classification

INRG STAGE	AGE (months)	Histologic Category	Grade of Tumor Differentiation	*MYCN*	11q Aberration	Ploidy	Pretreatment Risk Group
L1/L2		GN maturing; GNB intermixed					A Very low
L1		Any, except		NA			B Very low
		GN maturing or GNB intermixed		Amp			K High
L2	<18	Any, except GN maturing or GNB intermixed		NA	No		D Low
					Yes		G Intermediate
	≥18	GNB nodular; neuroblastoma	Differentiating	NA	No		E Low
					Yes		H Intermediate
			Poorly differentiated or undifferentiated	NA			
				Amp			N High
M	<18			NA		Hyperdiploid	F Low
	<12			NA		Diploid	I Intermediate
	12 to <18			NA		Diploid	J Intermediate
	<18			Amp			O High
	≥18						P High
MS	<18			NA	No		C Very low
					Yes		Q High
							R High

(adapted from S.L. Cohn, A.D. Pearson, W.B. London, T. Monclair, P.F. Ambros and G.M. Brodeur *et al.*, The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report, *J Clin Oncol* 27 (2009), pp. 289–297)