Neuroblastoma: Biology and Therapy

ABSTRACT: Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 15% of cancer-related deaths. These tumors have a predilection for young children; 60% of cases occur before age 2 years and 97% before age 10. Neuroblastomas derive from embryonic neural crest cells of the peripheral sympathetic nervous system. The behavior of this malignancy is characterized by marked clinical heterogeneity, ranging from spontaneous maturation in some patients to inexorable rapid metastatic progression in others. This article will discuss some of the molecular and biological features of neuroblastoma that are associated with these differences in behavior, and how these features have been used to develop a risk-based approach to therapy.


Introduction

Approximately 500 cases of neuroblastoma are diagnosed annually in the United States. Overall incidence from birth to age 15 is 8.7 cases per million per year. The disease has a striking predilection for young children; 60% of cases occur before age 2 years and 97% before age 10. Thus far, no definite environmental risk factors have been determined, although single studies have suggested a possible increased risk from maternal exposure to alcohol, neurally active drugs, diuretics, and hair coloring and from paternal exposure to electromagnetic fields. The clinical behavior of neuroblastoma varies markedly, ranging from spontaneous maturation in some patients to inexorable rapid metastatic progression in others. This article will explore some of the molecular and biological features of neuroblastoma that are associated with these differences in behavior and the use of these features to develop a risk-based approach to therapy.

Molecular Genetics and Cellular Markers

Chromosome Abnormalities

Deletion of the distal short arm of chromosome 1 was the first described genetic mutation in neuroblastoma tumors and is the most consistently reported abnormality.[1,2] Cytogenetic analysis of near-diploid neuroblastoma tumors and cell lines shows deletion of the distal short arm of chromosome 1 in 70% of cases. This deletion is found more frequently in patients with advanced disease and a poor prognosis. However, due to the technical difficulties inherent in karyotyping solid tumors, fewer patients with low-stage disease have been examined, although those tested have lacked the abnormality.

To eliminate the need for karyotyping, Fong et al studied DNA by comparing constitutional (lymphocyte) DNA to tumor DNA. They identified loss of heterozygosity at one or more loci on chromosome 1 in 13 of 47 tumors and showed, again, that this abnormality correlates with advanced, poor-prognosis disease.[1] Subsequent investigation revealed frequent deletions on chromosome 11 and chromosome 14, as well as rearrangements on chromosome 17.[3]

More recent screening studies for abnormalities of chromosome number have used comparative genomic hybridization. This technique summarizes DNA copy number abnormalities by mapping them to their positions on normal metaphase chromosomes, based on simultaneous hybridization of tumor and normal DNA labeled in different colors to normal metaphase spreads. The ratio of tumor to normal fluorescence intensities along the target chromosomes indicates relative increases and decreases in DNA copy number throughout the entire tumor genome. In a preliminary study of 29 cases from the Children’s Cancer Group (CCG), Plantaz et al showed that chromosome 17 gains were
the most frequent abnormality in neuroblastoma, seen in greater than 70% of cases.[4]

**MYCN Amplification**

One of the other intriguing abnormalities found in neuroblastoma tumors, which has been shown to correlate very strongly with outcome, is *MYCN* amplification, which is found in about 30% of tumors. The *MYCN* proto-oncogene is derived from the c-myc viral oncogene and is located on the distal arm of chromosome 2. The finding of *MYCN* amplification correlates with the cytogenetic abnormalities of double minutes (small extrachromosomal amplifications of *MYCN* DNA) and homogeneously staining regions (regions of *MYCN*-amplified DNA that can be integrated into any otherwise normal chromosome). FIGURE 1

Amplification of *MYCN* has now been shown to correlate very closely with advanced-stage disease and, within each stage, to be highly predictive of outcome.[5] *MYCN* may now readily be determined by traditional Southern blot DNA analysis, the technique of fluorescence in situ hybridization (FISH; Figure 1), immunostaining of tissues for protein expression, and the sensitive polymerase chain reaction (PCR), as well as by comparative genomic hybridization.[4-7]

Other genes commonly implicated in cancers, such as the *ras* family of p53, have not been commonly affected in neuroblastoma tumors.

**Ploidy**

Chromosomal ploidy is another marker of prognosis that has proved to be particularly useful in infants less than 18 months of age. Near-diploid or pseudodiploid tumors have near-normal nuclear DNA content but often have structural chromosomal aberrations, including *MYCN* amplification. In contrast, hyperdiploid or near-triploid tumors are most common in infants who have tumors that lack 1p deletion or *MYCN* amplification and have an excellent prognosis. A small subset of infants with diploid tumors have a much poorer prognosis than those with hyperdiploidy.[8]

**Cellular Markers of Differentiation**

Several cellular markers of differentiation along neuronal pathways have been shown to have prognostic importance in neuroblastoma, suggesting that the malignant transformation of these cells may result, in part, from inadequate response to the usual inducers of neuronal differentiation. Investigations have demonstrated abnormalities in the nerve growth factor receptors in neuroblastoma cell lines. These studies have shown a significant correlation between the increased expression of high-affinity nerve growth factor receptor (gp140TRK-A) and tumors with single-copy *MYCN* and a favorable outcome, as well as a correlation between lack of expression of the nerve growth factor receptors and both *MYCN* amplification and poor survival.[9]

**Telomerase**

Another determinant of malignant progression may be telomerase, an RNA-protein complex that prevents cell senescence by maintaining chromosomal telomeres. Telomerase is expressed in normal germ-line but not most somatic cells, and it appears to be necessary for immortalization of malignant cells. Telomerase activity has recently been reported to correlate with both *MYCN* amplification and stage in neuroblastoma.[10] We have now examined a large group of primary neuroblastomas for RNA levels of telomerase and have shown correlations with both stage and event-free survival, with
poorer event-free survival in patients whose tumors show higher telomerase expression.[11]

**Prognostic Factors and Risk Groups**

**TABLE 1**

Clinical Factors in Neuroblastoma Analyzed Singly

In addition to the many molecular genetic determinants of malignant progression, many more general laboratory, pathologic, and clinical markers have been shown to correlate with prognosis (Table 1). Shimada has developed a powerful classification system that combines age with histopathologic grading (based on the amount of neural stroma), mitotic-karyorrhectic index, and differentiation, as well as diffuse vs nodular appearance. This prognostic classification, which appears to be independent of MYCN amplification, has been verified in both retrospective and prospective CCG studies.[12,13] A number of serum markers have also shown to be prognostic for outcome, probably because, in part, they are surrogate markers of tumor burden. These include serum levels of lactic dehydrogenase (LDH), ferritin, GD2 ganglioside, and chromogranin. The most important clinical risk factors are disease stage and age at diagnosis, with age less than 1 year conferring the most favorable prognosis. Other clinical risk factors that may have some adverse importance include bone metastases, bone marrow metastases, and primary site in the abdomen (as opposed to the more favorable sites of the pelvis or thorax).[2,14-18]

**Screening and Diagnosis**

Given the high incidence of neuroblastoma in infancy, newborn urinary screening for catecholamines, excreted in 80% to 90% of cases, has been suggested as a cost-effective way to detect this tumor. Interestingly, the results of screening at birth and 6 months reported thus far suggest that most of the cases detected by this method are biologically favorable tumors with a high rate of spontaneous regression. Although screening has revealed an increased incidence in infancy, the survival or stage distribution of cases in children over age 1 year is not substantially affected, suggesting that this method does not detect the more malignant tumors at an early stage.[2] Approximately half of children who present with neuroblastoma already have disseminated disease. The most common symptoms are pain due to either primary tumor or to bone and bone marrow involvement, an abdominal mass, weight loss, anorexia, and irritability. Metastases to the orbit are common with resulting proptosis, while mediastinal primaries may be accompanied by Horner’s syndrome. Rare presentations of neuroblastoma due to paraneoplastic phenomena include secretory
diarrhea (due to secretion of vasoactive intestinal peptide) or opsoclonus, myoclonus, and ataxia (which is possibly due to cross-reacting antineuronal antibodies elicited by the tumor, which cause acute cerebellar encephalopathy and result in this syndrome in about 3% of patients).

Primary tumors arise in the abdomen in 70% of patients, often in the adrenal gland and next most frequently in the chest. Bone marrow and bone are the most common sites of metastases, while lung and central nervous system metastases are very rare (less than 5%).

Clinical Evaluation of Neuroblastoma

Diagnostic evaluation of neuroblastoma requires tissue for both diagnosis and prognosis, since histologic grading, as well as molecular genetic studies, are essential for management decisions. Urinary catecholamine determinations and serum ferritin and LDH levels are useful both in assessing prognosis and as a baseline for disease monitoring (Table 2).

Metastatic and staging evaluation includes, at a minimum, a computed tomographic (CT) or magnetic resonance imaging (MRI) scan of the primary, bone scan, bilateral bone marrow aspirate and biopsy, and, optimally, an iodine-123- or iodine-131-metaiodobenzylguanidine (MIBG) scan (Table 2). Meta-iodobenzylguanidine is a derivative of guanethidine, similar in structure to norepinephrine, that specifically concentrates in neuroblastoma and pheochromocytoma. It is 90% sensitive for tumor detection, and has the advantage of imaging equally well in soft-tissue, bone, and bone marrow tumors, with no uptake in normal bone, thus preventing the confusion with normal epiphyses that can occur with technetium scans.[19,20] Additional sensitivity for bone marrow metastases can be achieved with immunocytologic testing using either immunofluorescence or immunoperoxidase stain, which can detect as little as 0.001% tumor infiltration, including unsuspected bone marrow tumor in patients with otherwise low-stage disease.[21,22]

Staging Systems

Several different staging systems have been used to help plan treatment for neuroblastoma: (1) the original and widely used classification of Evans, also used by the CCG; (2) the St. Jude Children's Research Hospital system, now revised and utilized by the Pediatric Oncology Group (POG); and (3) the International Union Against Cancer (UICC) tumor-node-metastasis (TNM) system. All three systems have prognostic value. An international consensus group has arrived at a new synthesis of these systems, the International Neuroblastoma Staging System (INSS; see Table 3). The international system is being tested further prospectively in order to facilitate international comparisons of treatments.[19,20]

Risk-Based Therapy

Risk Classification

TABLE 4
Risk Groups for Neuroblastoma

Treatment of neuroblastoma must be tailored to both the extent of disease and the biological risk characteristics. A working division into risk groups, shown in Table 4, categorizes patients into three groups requiring different treatment intensities. Further study using multivariate analysis may result in greater refinement of a combined anatomic and biological staging system. However, the current working classification used by the CCG has succeeded in identifying those children who have an excellent prognosis when given little or no therapy and those who have a guarded prognosis even with very aggressive treatment.

Low-Risk Group

The low-risk group is comprised of patients with INSS stage 1, 2, or 4s disease and usually favorable biological characteristics. These patients require minimal therapy, usually surgery alone, unless they have local disease-related symptoms, such as cord compression from a paraspinal tumor or respiratory distress from liver infiltration by a tumor in the intraventricular system (Figure 2). FIGURE 2

Both retrospective and now prospective studies have confirmed that surgery alone is sufficient therapy for stage 1 and 2 neuroblastoma. A retrospective CCG analysis showed that the survival rate among patients with stage 2 disease was greater than 90% regardless of measurable postoperative residual tumor.[15] This has been confirmed in prospective studies conducted by both the POG and CCG. The overall survival rate for more than 200 children with stage 2 disease treated with initial surgical management is greater than 95% regardless of other biological features, with only 13% of patients requiring any chemotherapy for symptoms. Even the 15% of patients who have a local tumor recurrence after surgery can usually be treated effectively with moderate-intensity chemotherapy or
local radiation. [unpublished CCG data]
Neither radiation nor chemotherapy has been shown to contribute to survival in the low-risk group as a whole. The only exceptions are children over 1 year old at diagnosis who have stage 2 disease with tumor MYCN amplification. In previous studies, these children showed rapid progression and dissemination without aggressive therapy; therefore, it is now recommended that they receive very aggressive therapy on the treatment protocols for high-risk patients.
Children with stage 4s neuroblastoma also had a 90% overall 3-year survival in the most recently completed CCG study, CCG-3881. However, approximately half of these children required a short course of chemotherapy with cyclophosphamide (Cytoxan, Neosar), and some received hepatic irradiation for respiratory distress. The rare patient with MYCN amplification progressed rapidly, and therefore, will be treated as a high-risk patient in the future. [23,24]

Intermediate-Risk Group
The recently completed CCG-3881 study for intermediate-risk patients treated all infants with stage 3 disease; children over 1 year old with stage 3 disease and favorable Shimada classification, normal ferritin (less than 143 ng/mL), and single-copy MYCN; and infants with stage 4 disease and nonamplified MYCN with a 9-month course of chemotherapy, surgery, and local radiation to residual disease. The 3-year event-free survival rate for the stage 3 patients was 96%. Event-free survival did not differ between the infants and the children greater than 1 year old with favorable-biology tumors, nor between the infants with unfavorable biological features and those with no unfavorable biological features.
In the past, the subset of stage 3 patients greater than 1 year old at diagnosis had an event-free survival of only 30% to 40%. In CCG-3881, stage 4 infants had a 75% 3-year event-free survival overall, but if only patients with known nonamplified MYCN were considered (N = 72), the 3-year event-free survival was 95%.
The excellent outcome for these intermediate-risk patients has led to the goal of decreasing cost and late sequelae in this group by decreasing the overall cumulative dose and duration of therapy without compromising dose intensity. Thus, in the next intermediate-risk protocol (a combined effort of the CCG and POG), patients will be treated with only four cycles (12 weeks) of combination chemotherapy, all administered in the outpatient setting; the only exception will be children with either unfavorable Shimada classification or diploidy, who will receive eight cycles. The stage 3 and 4 patients with MYCN amplification will be considered high risk regardless of age, and will be treated on a different protocol. [16,23,24]

High-Risk Disease

The high-risk group consists mainly of patients with stage 4 disease who are greater than 1 year old at diagnosis, but also includes the following subgroups: stage 3 children greater than 1 year old with either MYCN amplification or unfavorable Shimada features; stage 2 patients greater than 1 year with MYCN amplification; and stage 3, 4, and 4s infants with MYCN amplification. These patients continue to respond poorly even to very dose-intensive therapy, although incremental advances have certainly been made over the past decade.
As shown in Figure 3, the 4-year survival rate among the 507 stage 4 patients greater than 1 year old who were treated in the CCG studies from 1978 to 1985 was 9%, as compared with a rate of 30% among the 675 patients treated from 1991 to 1995 (P < .001). [24] Some of this improvement may be attributable to increases in the dose intensity of therapy; a meta-analysis by Cheung and
co-workers has shown an effect of dose intensity in neuroblastoma similar to that reported in other cancers.[25] The improvement may also be the result of the increasing use of high-dose myeloablative therapy with autologous or allogeneic bone marrow transplantation (BMT).[26-33] Nonrandomized Studies—Between 1988 and 1993, the CCG completed three concurrent studies of high-risk neuroblastoma: CCG-321P1, which tested allogeneic BMT; CCG-321P2, 1 year of chemotherapy; and CCG-321P3, autologous purged BMT. All three studies treated patients with five to six cycles of induction chemotherapy consisting of cisplatin (Platinol), doxorubicin, cyclophosphamide, and etoposide (VePesid). After undergoing surgery for local control and receiving irradiation to residual disease sites, patients received ablative chemotherapy and total-body irradiation (TBI) with BMT or were continued on four-drug induction chemotherapy for a total of 13 cycles.

Several important conclusions can be drawn from these nonrandomized studies:

1. Allogeneic BMT was not shown to be superior to autologous purged BMT and, in fact, had a higher toxic death rate and an equal relapse rate.[29]
2. With a median follow-up of 5 years, the event-free survival at 5 years from the time of autologous BMT for 147 patients is 37%. FIGURE 4

3. In a retrospective nonrandomized comparison of all of the stage 4 patients who continued to receive chemotherapy vs those who went on to receive purged autologous BMT, the event-free survival rate was significantly better for those receiving autologous BMT (40% vs 19%; Figure 4).[18] This retrospective analysis involved 207 children greater than 1 year at diagnosis with stage 4 neuroblastoma treated on CCG protocol 321P2 (described above). By the end of five to seven courses of chemotherapy, 159 patients were event-free; 67 of these patients went on to receive autologous BMT, while 74 continued chemotherapy for a total of 1 year. The relative risk of an event after autologous BMT was only 58% of that with chemotherapy (P = .01). An even more significant advantage for autologous BMT was seen among patients who were only in partial, as opposed to complete, remission at the end of induction and for those who with tumor MYCN amplification.

Other Comparisons of Autologous BMT and Chemotherapy—Previous smaller studies comparing autologous marrow transplantation to chemotherapy had yielded conflicting results, with Pinkerton reporting the only randomized trial showing a significant improvement with autologous BMT.[32] This report included a total of only 65 patients, however. A retrospective comparison by the POG of 116 patients achieving complete or partial remission did not show any significant difference in outcome for the 32 patients who underwent BMT prior to progression.[34] The Study Group of Japan reported a 50% event-free survival rate in a nonrandomized group of patients given myeloablative therapy with autologous BMT, as compared with a 39% rate in those who received chemotherapy.[31] Overall, autologous BMT appears to be at least as effective as intensive chemotherapy and may provide an advantage in some extremely high-risk subgroups. However, a definitive conclusion cannot be made until the results of a recently completed CCG randomized prospective trial comparing outcomes among unselected high-risk patients from the time of diagnosis become available.

Another ongoing phase I dose-escalation study of a non-TBI-containing myeloablative regimen has thus far shown event-free survival comparable to that seen in the previous 321P3 study (55% at 2 years from autologous BMT). This phase I study has further verified the earlier suggestion from Kushner and co-workers[28] that higher-dose local irradiation to tumor sites may prevent primary site relapse, a common problem in previous CCG protocols. However, the relapse rate for patients transplanted after first disease progression has continued to be high; the current event-free survival rate at 2 years post-transplantation is only 15%. This is similar to the poor outcome observed in European studies, as reported by Ladenstein and colleagues.[27]
Recently Completed and Ongoing Trials

CCG Trial in High-Risk Disease

The most recently completed CCG trial in high-risk disease was designed to answer, in a prospective, randomized fashion, the relative importance of ablative therapy with purged autologous BMT vs intensive consolidation. The study accrued 550 high-risk patients in 4-1/2 years, with approximately one-third of patients randomized to each arm and the other one-third nonrandomly assigned to consolidation chemotherapy if they refused to be randomized. A second randomization, done at the end of chemotherapy consolidation or autologous BMT, assigned patients to receive or not to receive 6 months of 13-cis-retinoic acid (isotretinoin [Accutane]). In vitro and some in vivo studies have reported this differentiating agent to have efficacy against neuroblastoma. The outcome of this study, still blinded for analysis, will help determine the future role of myeloablative therapy, as well as the potential utility of differentiating agents, in high-risk neuroblastoma. Current and future studies will examine repetitive stem-cell transplants, more intensive induction therapies, and the use of alternative stem-cell sources.

Source and Purity of Stem Cells for Ablative Therapy

The source and purity of the stem cells used for ablative therapy in neuroblastoma have been the focus of concern. Immunocytology techniques have shown that as many as 70% of patients have bone marrow tumor at diagnosis and 50% have circulating tumor cells in peripheral blood. Even after several cycles of induction chemotherapy, 25% of patients still have some detectable tumor by bone marrow immunocytology and 7% have circulating tumor cells.[22] Such cells in bone marrow have the potential for tumorigenicity. This has been inferred from studies showing the development of tumor cell lines from harvested bone marrow and by reports of miliary lung metastases following autologous BMT,[35] and has been demonstrated more definitively by the gene-marking studies of Rill and co-workers.[36] In these gene-marking studies, autologous unpurged but histologically tumor-free marrow was transfected with the neomycin resistance gene and then reinfused into patients, some of whom subsequently relapsed with neuroblastoma tumors expressing the marker.

More recently, testing of peripheral blood stem-cell collections and bone marrow with PCR has confirmed the problem of contamination with tumor cells, even in CD34-selected preparations. The significance of such low-level contamination is still undetermined but will be examined in future studies. Such contamination suggests the need for effective methods to purge both peripheral blood stem cells and bone marrow prior to reinfusion.[37]

Novel Therapeutic Approaches for Metastatic Disease

Dose-intensive therapy, with or without stem-cell support, is rapidly approaching the limits of toxicity, both in terms of acute and late effects. Novel approaches that are more tumor-specific and less toxic are required to make further progress in metastatic neuroblastoma. Approaches under investigation that show some promise in the laboratory and in preliminary phase I and II studies include immunologic modulators, including tumor-specific antibody, antibody given with granulocyte colony-stimulating factor (GM-CSF, filgrastim [Neupogen]) or interleukin-2 (IL-2 [Leukine]), fusion proteins of antibody with cytokines, and tumor vaccines; tumor-targeted therapy utilizing radiolabeled antibody or MIBG; and tumor-differentiating agents.

Immunologic Modulators

Phase I and II studies of anti-GD2 antibodies have shown some modest antitumor efficacy in patients with relapsed neuroblastoma.[38,39] Both the murine monoclonal 3F8 and 14G2a (murine), the latter tested with IL-2 and with GM-CSF, have shown some tumor responses, as well as in vitro stimulation of antibody-dependent cellular cytotoxicity (ADCC). More recently, Yu and co-workers have completed a phase II trial of the human chimeric anti-GD2 antibody, CH14.18 with GM-CSF, which again shows some responses, most frequently in bone marrow tumor.[40]

Targeted Therapy

Targeted therapy using antibody or MIBG for the delivery of radiation in the form of iodine-131 has also been tested in clinical trials. Cheung and co-workers have documented responses to iodine-131-3F8 among patients with refractory neuroblastoma,[41] and are currently conducting a
study in which newly diagnosed patients are receiving iodine-131-3F8 in ablative doses followed by bone marrow rescue, with further treatment with cold antibody post-transplant. Iodine-131-MIBG has been widely tested in Europe for refractory neuroblastoma and, more recently, for initial therapy in patients with regional disease. We have conducted a phase I dose escalation trial of iodine-131-MIBG at the University of California (San Francisco) and have determined the maximal non-marrow-ablative dose and the maximal practical ablative dose with stem-cell rescue.[42] In 30 patients, we observed a 37% response rate and no significant toxicity other than hematologic effects. Future studies will utilize combinations of ablative chemotherapy with iodine-131-MIBG.

**Differentiating Agents**

Differentiating agents are another approach for circumventing the undesirable effects of cytotoxic agents. For many years, laboratory studies have shown the capacity for either spontaneous or induced differentiation and growth arrest of neuroblastoma cell lines in culture. Currently, various derivatives of retinoic acid are the differentiating compounds in clinical testing. One such derivative, 13-cis-retinoic acid, has been reported to cause remissions in refractory patients, and a recently completed phase II trial showed a 10% response rate in relapsed neuroblastoma.[43-45] The recently completed randomized CCG-3891 study randomized all patients at the end of consolidation therapy to receive 13-cis-retinoic acid or no further therapy; results of this trial are pending.

Other retinoids currently in phase I investigation include all-trans-retinoic acid (ATRA [Vesanoid]) combined with interferon-alfa (Intron A, Roferon-A), 9-cis-retinoic acid, and fenretinide, another analog that causes growth arrest and apoptosis in vitro, even in cell lines shown to be resistant to trans-retinoic acid.

**Conclusions**

Substantial advances are occurring in the elucidation of the molecular pathogenesis of neuroblastoma, as well as in the definition of clinical prognostic groups. In addition, modest but significant improvements in the treatment of metastatic disease have been achieved by increasing dose intensity with the use of hematopoietic support. However, in order to overcome chemotherapy resistance, new therapeutic approaches are required using tumor-targeting, differentiating, or apoptotic agents; stimulation of host immune response; or genetic manipulation.

**References:**


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