In Situ Neuroblastoma: The Intriguing "Tumor" of Neuroectodermal Origin and the Putative Cancer Stem Cells

Consolato M. Sergi[1,2]

[1]Anatomic Pathology Division, Children's Hospital of Eastern Ontario (CHEO), University of Ottawa, Ottawa, ON, Canada; [2]Department of Laboratory Medicine and Pathology, University of Alberta, Edmonton, AB, Canada

Address for Correspondence: Consolato M. Sergi, Anatomic Pathology Division, Children's Hospital of Eastern Ontario (CHEO), University of Ottawa, Ottawa, ON, Canada. Email: csergi@cheo.on.ca

Abstract: Neuroblastoma is the most prevalent solid tumor encountered outside of the central nervous system in infancy. It is considered an embryonal tumor arising from pluripotent sympathetic cells called neuroblasts with characteristic neurosecretory granules. They are positive for chromogranin A, and are double membrane bounded ultra-structurally. In rare occurrences, it can be identified at neonatal age or at very early infancy. *In situ* labelling of neuroblastoma has been used, but there is a remarkable controversy around it, because most of these clusters of neuroblastic cells do not exhibit a neoplastic potential. These clusters often regress, and these pediatric patients should not receive chemotherapy. This chapter reviews the challenges of this tumor and report on the heterogeneity of this kind of pediatric neoplasm with a focus on putative cancer stem cells.

INTRODUCTION

Neuroblastoma is the most prevalent tumor encountered outside of the central nervous system in infancy and fine-needle aspiration biopsy has been advocated for the diagnosis (1, 2). It is considered an embryonal tumor arising from pluripotent sympathetic cells called neuroblasts with characteristic neurosecretory granules, which are positive for chromogranin A and double membrane bounded ultra-structurally (3). During embryogenesis, the neuroblasts invaginate and migrate along the neuraxis. Then, they populate the adrenal medulla, the sympathetic ganglia, the Meissner (*submucosus*) and the Auerbach (*myenteric*) plexuses of the gastrointestinal tracts as well as other sites (1). Relevant prognostic factors in pediatric oncology are age, stage, and biological features identified in tumor cells. These characteristics are key for the risk stratification and the assignment of treatment protocols (1).

Neuroblastoma has an incidence of 10.5 per million in children under 15 years of age, but the rates keep increasing and the role of pesticides and insecticides during blastogenesis or the parental exposure has been recently suggested (4–6). In pediatric oncology, it accounts for 8–10% of all pediatric neoplasms, and for about 15% of cancer death in pediatrics (1, 7, 8). There is no significant geographical variation in the incidence between North America, Europe, and Australia. Despite there are also no differences in different ethnicities, neuroblastoma seems to be more frequent in boys than in girls (1.2:1 ratio). Although the peak incidence is between 0 and 4 years of age, neuroblastoma can occur also in later childhood and even adult cases have been described in the literature and the expression of cancer antigens are not limited to childhood (9). There are familial cases of neuroblastoma, but they are exceedingly rare. Apart from parental exposure to pesticides and insecticides, paternal exposure to electromagnetic fields or prenatal exposure to alcohol or phenobarbital have been listed as environmental factors (4). In infancy, the measurement of urinary catecholamine metabolites is a characteristic diagnostic procedure (1).

PATHOLOGY

Neuroblastoma is a peripheral neuroblastic neoplasm, which belongs to the group of small round blue cell tumors of childhood (1) (Figure 1). Neuroblasts arise from progenitor cells of the sympathetic nervous system, which are also known as the sympathogonia of the sympathoadrenal lineage. Migrating from the neural crest of embryogenetic time, these cells build the sympathetic ganglia, the paraganglia, and the chromaffin cells of the adrenal medulla, thus reproducing the typical sites of neuroblastic neoplasms. It has been presumably indicated that defects in embryonic genes regulating the development of the neural crest are probably the underlying disorders causing the proliferation of halted stages of neuroblastic cell differentiation (10, 11). In embryonal tumorigenesis,
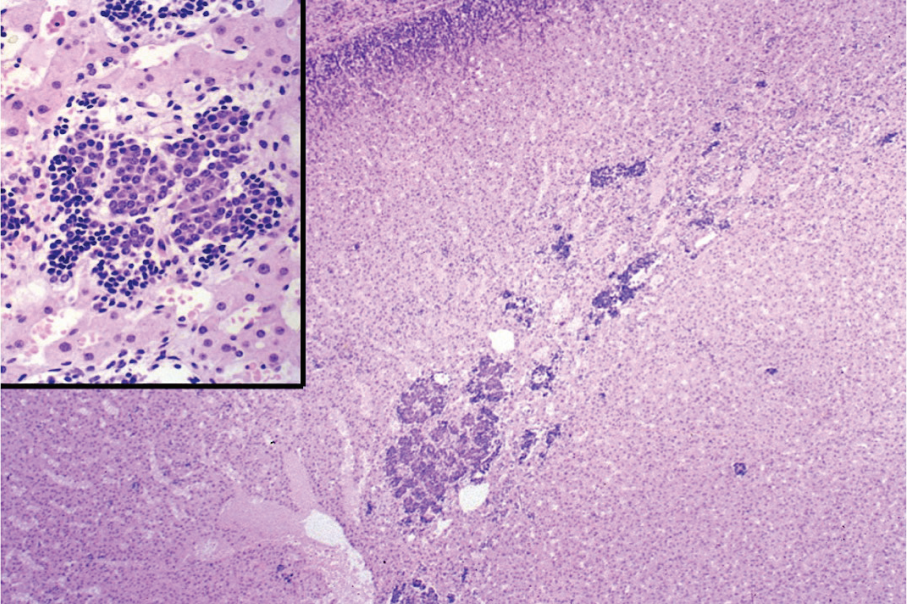
Figure 1. **Neuroblastoma.** Photomicrograph showing an overview and a high-power magnification of an in situ neuroblastoma illustrating the undifferentiated small round blue cell tumors in clusters exhibiting a high nucleus-cytoplasm ratio. The image is from the Neuroblastoma Atlas by the International Neuroblastoma Pathology Committee. Courtesy of Professor Hiroyuki Shimada, Department of Pathology, Stanford University, CA, USA.

developmental programs regulating self-renewal in neuronal stem cells, including the Wnt/Beta catenin, Notch, and Sonic hedgehog have been involved (1). In fact, Zhi et al. explored the role of Wnt/Beta-catenin in the modulation of the plasticity of the N2A cells-derived neurons and speculated the origin of neuroblastoma (12). These authors found that beta-catenin in its activated form was up-regulated and in pace with the clinical risk of neuroblastoma. The Hedgehog (HH) signaling pathway has been targeted by Wickström et al. (13). The use of specific inhibitors disclosed that inhibiting the HH signaling pathway at the level of GLI was most effective in decreasing the growth of this tumor. In addition, GANT61 sensitivity correlated with GLI1 positively and MYCN expression negatively in the tested neuroblastoma cell lines. These, and studies targeting the FOXO pathway have not yet modified the clinical practice or therapeutical strategy substantially (10, 11, 14–16).

THE QUEBEC AND GERMAN NEUROBLASTOMA SCREENING PROGRAMS

The International Neuroblastoma Pathology Classification (INPC) have identified the following four basic categories: (i) neuroblastoma (Schwannian stroma-poor); (ii) ganglioneuroblastoma, intermixed (Schwannian stroma-rich); (iii) ganglioneuroma

(Schwannian stroma-dominant); and (iv) ganglioneuroblastoma, nodular (composite Schwannian stroma-rich/stroma-dominant and stroma-poor). The current classification ideated by Shimada et al. in 1984 stratify patients optimally using the age of the patient, the degree of neuroblast differentiation, the lack of Schwannian stromal development (stroma-poor vs. stroma-rich), the index of cellular proliferation with a singular index grouping both mitoses and karyorrhectic figures, the so-called MKI or mitosis-karyorrhexis index, and the pattern associated to nodularity (17). Briefly, two major categories of neuroblastoma patients have been identified. Patients with low-risk and intermediate-risk harbor excellent prognosis, while patients with high-risk disease disclose poor outcome rates despite intensive programs of chemotherapy. Four out of five patients aged 18 months or older show metastatic disease at presentation. This neuroblastoma presentation may include liver, bone, bone marrow, and lymph nodes. These categories have been implemented by additional information derived by molecular pathology. *MYCN* amplification is currently the most critical biomarker in patients suffering from neuroblastoma. The oncogene labelled *MYCN* is in the distal arm of chromosome 2 and amplified in one-fourth of de novo patients and is more often found in advanced stages of the disease. Additional biomarkers may also include *H-ras* expression (an oncogene, which correlates with lower stages of neuroblastoma), chromosome 1 deletion (this region has several tumor suppressor genes that control the neuroblastic differentiation), DNA index (a value greater than one indicating hyperdiploidy harbors good chemotherapeutic response, while a value lower than one indicating hypodiploidy necessitates a more aggressive chemotherapeutic approach), neurotrophin receptors (*TrkA* and *TrkC* genes expression are inversely correlated to the *MYCN* amplification, while *TrkB* is more often found in direct agreement of the *MYCN* amplification), apoptotic pathways disruption, CD44, and the multidrug resistance protein (MRP), but the last three seems to be controversial.

Very few screening programs have been established for the search of neuroblastoma at early stage. The Quebec Neuroblastoma Screening Project and the German Neuroblastoma Screening Program are the most important and verified screening programs used. These tests have been used in the past to identify this pediatric tumor at an early stage with the intent to discover it with a size amenable to be resected or starting chemotherapy protocols harboring a high success rate. Both programs demonstrated that neuroblastoma screening at age 1 or lower identifies more of this kind of pediatric neoplasms. However, they also indicated that these neoplasms, despite the increased incidence rates, harbor a good prognosis. Most importantly, and probably crucial for public health measures, these programs fail to detect the poor prognosis harboring neuroblastoma that presents clinically at an older age.

HETEROGENEITY CONUNDRUM AND THE *IN-SITU* NEUROBLASTOMA

Cancer stem cells (CSCs) have been considered the source of heterogeneous non-tumorigenic cell population of the neoplastic processes. Intratumor and intertumoral heterogeneity is now well-established, and they bring in huge challenges in

the diagnosis and treatment of neoplasia. The literature seems to suggest that heterogeneity arises gradually as tumor-initiating (carcinoma) stem cells (18). They harbor genetic and/or epigenetic changes that permit them to serially separate into multiple tumor cell types. There are multiple studies supporting the paramount importance of CSCs. Bergsagel and Valeriote (19) recognized that an *in vivo* assay for colony formation is critical in identifying a small fraction of neoplastic cells that could build a tumor colony showing a low proliferative rate. Equally, Park et al. (20), in a colony-forming experiment, have revealed that only as few as 1 in 10,000 purified rodent myeloma cells could build colonies. Other studies revealed the importance of the CSC in acute myelogenous leukemia (AML) (21). It seems that very few neoplastic cells with a specific phenotype have the ability for self-renewal. These cells have the capability to initiate tumors in non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) or NOD-SCID mice. Immunophenotypically, these cells are CD34+, CD38–, while the majority of the neoplastic cells present are a CD34–, CD38+. Likewise, Hamburger and Salmon demonstrated that in solid tumors, such as lung and ovarian cancer, 1 in 1,000 to 1 in 5,000 of the neoplastic cells disclose the potential to start colonies in soft agar assays (22).

Overall, these studies seem to consolidate the hypothesis that there is unquestionably a small cellular population that can support the consensus of tumor cell heterogeneity. In origin, it was alleged that neoplastic cells that constitute the majority of the tumor were homogenous. It was thought that each neoplastic cell could generate a new form of neoplasm as well as the option to gain stem cell-like assets (20, 21, 23–25). Subsequently, with the documentation of CSCs with a not yet fully defined cellular hierarchy, the idea of a stochastic model has now become inadequate. Data are consolidating the CSCs Model over the Stochastic Model. In fact, not all cancer cells have the possibility to initiate cancer, but a considerable number of cancer cells, which must include CSCs, are required to initiate tumor formation in immunocompromised mice. This setting, in turn, suggests that more cells are needed to start tumors because CSCs make up only a small portion of cancer. In 1982, Pierce et al. showed that embryonic tissue might harbor the capability to both inhibit tumor growth and impose CSCs to differentiate to cancerous cells (26). This investigation proposes the concept of "differentiation therapy". In consideration of this theory, CSCs may differentiate under specific circumstances (27). Thus, embryonic stem cells and their microenvironment could modify how CSCs originate and, more importantly, how they may be eliminated to achieve an enhancement in therapy. Several compounds, such as retinoic acid, have been recognized to have the capability to start cells to differentiate. Such compounds may sensitize tumors to chemo- and/or radiotherapy (27, 28). Further, the identification of several oncogenes, signaling pathways, and transcription factors that seem to be deregulated in CSCs have brought several scientists to think there may be new approaches for cancer therapy (29, 30).

Notch signaling is an example, in which over-activated CSCs may play a role. Therapeutical regimens that target Notch expression could evolve to the annihilation of CSCs (27, 31–33). Fan *et al.* (33) demonstrated that blocking the Notch signaling using γ-secretase could deplete neural (brain) CSCs and eradicate tumorigenic potential. For neuroblastoma, another target is the oncogene, *MYC*. This oncogene has been demonstrated to be paramount in upholding the uniqueness of sarcoma stem cells. *MYC*-inactivated sarcoma stem cells differentiate into

mature bone cells (osteocytes) (34). Moreover, CSCs have a DNA-repair mechanism and express drug-resistance proteins such as ABC family transporters (35). This property may explain the aptitude of CSCs to escape from chemotherapy and, consequently, trigger the recurrence of cancer. Thus, chemical compounds that inhibit ABC transporters in CSCs may give a better outcome comparing several chemotherapy regimens (36).

Neural crest cells (NCCs) aimed for the sympathoadrenal lineage can slowly migrate into the adrenals building Schwann Cell Precursors (SCPs). These cells can remain attached to the peripheral nerves. NCCs give origin to sympathoblasts, while SCPs give origin to both sympathoblasts and chromaffin cells. Neuroblastoma arises due to an arrest of differentiation during the normal development. It may be speculated that differences in cell progenitors (NCCs/SCPs) can be the cause of intertumoral variation, but dedifferentiation events in the neoplastic cells, which may not be uncommon as identified in IARC-based epidemiological studies (4, 5, 37), can drive the cell population to an early progenitor state (38). This dedifferentiation may lead to the intratumoral heterogeneity that we encounter in grossing and microscoping neuroblastomatous neoplasms of adrenal gland and peripheral nerve system as well as teratomas (39). Chromosomal instability is key in the clonal evolution of neuroblastoma because of the high rate of multiple somatic copy number changes identified in numerous studies. Also, epigenetic changes also contribute to clonal evolution of neuroblastoma leading to the emergence of events ultimately identifiable as major driver mutations during tumor progression. Thus, high-risk tumors may display features of undifferentiated cells such as bridge cells and chromaffin cells, while low-risk tumors have a higher amount of tumor cells that look like dedifferentiated sympathoblasts. On the other side, neuroblastoma is a complex neoplasm, and it is difficult to fully categorize it as CSCs-derived. According to the CSCs model, neoplasms arising from CSCs should disclose a hierarchical organization, which has not been the case with neuroblastoma yet. However, several investigations based on the expression of CD133, CD44, *c-KIT*, and *SOX-10* have identified a subpopulation of CSCs in neuroblastoma. In the future, such markers may be included in histopathology reports aimed at clinicians to improve the Shimada classification. Although there is no concrete proof that neuroblastoma originates from CSCs, the differentiation events induced by external factors and the multipotent cells like NCCs make neuroblastoma disclose a "stemness" behavior. The therapeutic targeting of some signaling pathways, such as Akt/mTOR, MAPK, and STAT, should be of benefit for high-risk tumors. Finally, the heterogeneous behavior of neuroblastoma needs to be examined also in terms of microenvironment, which may influence the clonal evolution and the dynamic regulation of CSCs as seen in cardiac myxomas and other tumors (40).

The incidence of in situ neuroblastoma has been up to fifty times the expected rate of primary adrenal neuroblastoma in children aged 15 years or younger. There is a frank concern that in situ neuroblastoma does not harbor a neoplastic potential (38, 41–45). There is also no proof that a clonal proliferation of genetically abnormal cells can originate in these lesions. Beckwith and Perrin indicated that probably very few of the *in-situ* neuroblastoma subsequently express a neoplastic phenotype (46). In Quebec, Canada, during the mass screening program by assessing vanillylmandelic acid (VMA) and homovanillic acid (HVA) in infants

between three weeks and six months, the rate of neuroblastoma cases peaked up to three times without any significant decrease of mortality in this pediatric age group (47, 48). The concept of dormant rests is well known for nephrogenic rests of renal development and nephrogenic rests can be identified in nephroblastoma independently of favorable or unfavorable histology (49). Peripheral neuroblastic tumors can evolve to neuroblastoma, ganglioneuroblastoma, and ganglioneuroma, but they also may involute and/or mature. In agreement with the International Neuroblastoma Pathology Classification, which separates favorable from unfavorable histology groups, the process of involution, also known as regression, is linked to a favorable group. Shimada argues that all microphotographs of the Beckwith and Perrin's contribution disclose morphologic features of a favorable histology category (50).

CONCLUSION

Currently, low-risk neuroblastoma is usually treated with surgery only and chemotherapy is used as an effective salvage therapy for individuals who relapse after surgery. The surgical approach and moderate-dose, multi-agent therapy (cyclophosphamide, doxorubicin, carboplatin, etoposide) are reserved for patients with intermediate-risk neuroblastoma. Radiotherapy is considered if residual disease occurs after surgery and chemotherapy. High-risk patients with neuroblastoma require surgery, multi-agent-chemotherapy, and radiation. Four phases of therapy have been established, including induction (alkylating agents, platinum, anthracyclines, and inhibitors of topoisomerase II), local control, consolidation (myeloablation using etoposide, carboplatin, and melphalan), and targeting the minimal residual disease. It is currently clear that neuroblastoma is one of the most heterogenous neoplasms of small round blue cell tumors. There is substantial evidence that most of the *in-situ* neuroblastoma do not progress. The final target of the heterogeneity of neuroblastoma will be a challenge, but the current technology is helpful as suggested for epigenomics and single cell sequencing studies of several tumors, including hepatoblastoma. The bequest of Beckwith and Perrin's classic contribution is a motivation for the future of young investigators to sit on the shoulders of giants. Here, creative thoughts may originate by re-reading old literature and implementing some ideas with new concepts originating from the most recent technological advancements as recently identified by Donato et al. (51) , who are applying artificial intelligence for studying the neuroblastoma.

Conflict of Interest: The author declares no potential of interest with respect to the research, authorship, and/or publication of this chapter.

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