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Pathogenesis and treatment of neuroblastoma

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BOSTON UNIVERSITY
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Thesis

PATHOGENESIS AND TREATMENT OF NEUROBLASTOMA

by

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B.S., Tufts University, 2016

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LEIDY ORTIZ

ABSTRACT

Neuroblastoma (NB) is the most common extracranial solid tumor that occurs in children. NB is highly metastatic and varies in risk level according to tumor histology and genetic characteristics. The amplification of the MYCN oncogene is one of the key factors that promotes the development of tumors and is associated with poor prognosis. Mutations in the tyrosine kinase ALK gene have also been linked to tumorigenesis, especially in familial neuroblastoma. Other genetic mutations that have been identified include ATRX, PTPN11, ARID1A, ARID1B, and genes involved in neuritogenesis. Genetic aberrations like chromothripsis, the loss of chromosome segments 1p and 11q, as well as the gain of segment 17q may also be linked to the development of NB. Treatment for this disease varies according to the patient's risk profile but may include surgery, high dose chemotherapy with or without bone marrow transplantation, and radiation. Several immunotherapy drugs, as well as drugs that target the disease pathway, are currently in development with the goal of improving treatment, survival rates, and quality of life. This review will discuss the molecular and genetic factors that drive neuroblastoma. It will also discuss the current treatments and survival rates of the disease as well as recent and ongoing treatment research.

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LIST OF ABBREVIATIONS

ALK.....	Anaplastic Lymphoma Kinase
ATM.....	Ataxia-Telangiectasia Mutated
AURKA.....	Aurora Kinase A
AURKB.....	Aurora Kinase B
AZA.....	5-Aza-deoxycytidine
BDNF.....	Brain Derived Neurotrophic Factor
CADM1.....	Cell Adhesion Molecule 1
CDH1.....	E-Cadherin
DAG.....	1,2-Diacylglycerol
DUSPs.....	Dual Specificity Phosphatases
EFS.....	Event-free Survival
EGFR.....	Epidermal Growth Factor Receptor
EMT.....	Epithelial Mesenchymal Transition
ER α	Alpha Estrogen Receptor
FACT.....	Facilitates Chromatin Transcription
FAN1.....	Fanconi-associated Nuclease 1
FANCM.....	Fanconi Anemia Complementation Group M
FZ.....	Frizzled Receptors
GDP.....	Guanosine Diphosphate
GM-CSF.....	Granulocyte-macrophage Colony-stimulating-factor
GTP.....	Guanosine Triphosphate

HBP1.....	HMG-box Transcription Factor 1
HDAC8.....	Histone Deacetylase 8
HLA.....	Human Leukocyte Antigen
INRG.....	International Neuroblastoma Risk Group
INSS.....	International Neuroblastoma Staging System
IP3.....	Inositol 1,4,5-triphosphate
L1-CAM.....	L1 cell adhesion molecule
MAPK.....	Mitogen-activated Protein Kinase
MDR1.....	Multi-drug Resistant Protein
miRNA.....	Micro-RNA
mRNA.....	Messenger RNA
mTORC1.....	Mammalian Target of Rapamycin Complex 1
NB.....	Neuroblastoma
NCI.....	National Cancer Institute
NGF.....	Nerve Growth Factor
NRAS.....	Neuroblastoma RAS
OS.....	Overall Survival
PCP.....	Planar-cell-polarity
PI3K.....	Phosphoinositide 3-kinase
PIP ₂	Phosphatidylinositol (3,4)-bisphosphate
PIP ₃	Phosphatidylinositol (3,4,5)-triphosphate
PKC	Protein Kinase C

PLCy..... Phosphoinositide Phospholipase Cy
PTPN11..... Tyrosine-protein Phosphatase Non-receptor Type 11
ROCK.....Rho-associated Kinase
SPRYs..... Sprouty Proteins
SSTR.....Somatostatin Receptor
VEGF.....Vascular Endothelial Growth

INTRODUCTION

Neuroblastoma is one of the most prevalent solid tumors in children and accounts for 15% of pediatric cancer deaths in the United States (Kim and Chung)¹. The disease usually affects children under the age of 5 and is most common in children under the age of 1, though teens and adults can also be affected. Survival rates for NB vary according to several risk factors and age at the time of diagnosis. Overall survival for infants under 18 months of age is 88%, 49% in children between 18 months and 12 years of age, and only 12% in adolescents and adults.^{2,3,4} Currently, there is no standardized screening for neuroblastoma. Tumors may often form before birth and may not be found until they have progressed to a point in which they cause symptoms. The majority of NB cases occur sporadically, but according to the National Cancer Institute (NCI) 1% to 2% of cases are genetic or familial in nature.⁵ Genetic cases follow an autosomal dominant inheritance pattern with incomplete penetrance⁶, meaning that not everyone who carries the mutation will develop the disease. Due to the rarity of familial neuroblastoma, this review will mainly focus on the pathogenesis and treatment of sporadic cases.

NB tumors typically appear in the abdominal region, but they can emerge on any axial level of the sympathetic chain as seen on figure 1. Approximately half of these abdominal tumors manifest in the adrenal glands.⁷ Regardless of their location on the body, tumor cells seemingly have a common embryological

origin, as they are derived from neural crest cells. During embryogenesis, neural crest cells migrate towards different areas of the body in order to give rise to various cells and structures, including the adrenal glands, melanocytes, facial bone, and the nervous system. The widespread migration of neural crest cells and their association with the onset of neuroblastoma may explain why tumors have the potential to appear on several axial levels of the torso.

NB is largely heterogeneous, as cases can have a variety of clinical outcomes. Lower risk tumors can regress or differentiate spontaneously into a benign ganglioneuroma.⁸ Some moderate and high-risk tumors may respond to treatment, while others may become resistant and ultimately lead to death. Several genetic mutations and alterations that play a role in NB pathogenesis have been identified. Some of these factors include, but are not limited to, the amplification of the MYCN oncogene, mutations in a tyrosine kinase receptor known as anaplastic lymphoma kinase (ALK), genetic aberrations involving the loss or gain of alleles, chromothripsis, and neuritogenesis genes. Among these, studies have demonstrated that MYCN is consistently the most important factor when determining risk level, as MYCN amplified tumors are especially aggressive.⁹ Activating ALK mutations have proven to be the main cause of familial cases of neuroblastoma, although mutations in this gene can also be found in sporadic NB.⁶

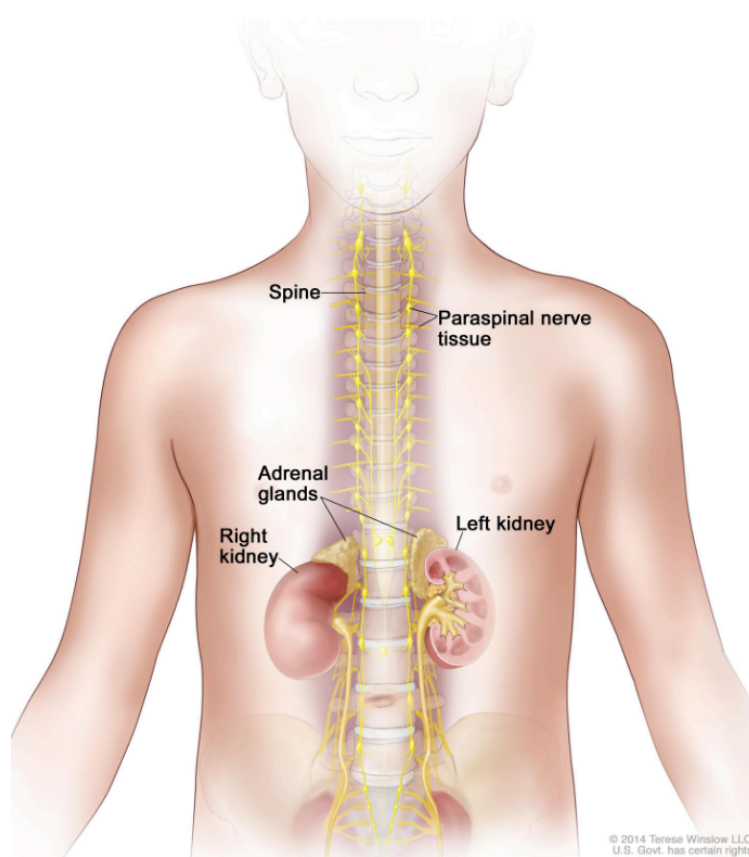


Figure 1. The Sympathetic trunk. Neuroblastoma can arise along any level of the sympathetic chain as shown. Most neuroblastomas arise in the abdominal sympathetic nerve ganglia, half of these are associated with the adrenal glands. Image taken from the National Cancer Institute's Physician Data Query on Neuroblastoma Treatment.⁵

A major focus for researchers is the regression and spontaneous differentiation of NB, as it may lead to greater understanding of the disease and the development of possible therapies. TrkA expression has been linked to the tumor's ability to spontaneously regress or progress. Tumors expressing TrkA/NTRK1 show a favorable prognosis.^{10,11} In contrast, tumors that express TrkB/NTRK2 or brain-derived neurotrophic factor (BDNF) are associated with unfavorable prognosis.¹²

The current treatment options focus on three approaches to treating tumors. These include surgical removal of the tumor, chemotherapy or radiation, and a combination of these therapies.⁵ Like other types of cancers, NB can metastasize and become challenging to treat by spreading to the liver, lungs, skin, and bone.¹³ Current knowledge on prognostic factors, like ALK and MYCN amplification, provides clues for potential pathways that can be exploited in order to find new and improved treatment for patients. Several clinical trials are currently being held with the goal of finding an effective targeted therapy for the disease.

SYMPTOMS AND STAGING

Common Symptoms

Neuroblastoma tumors are often present at birth and continue to grow in childhood. Nearly 20% of cancers are diagnosed within the first year of life and can be detected in the womb.¹⁴ Tumors most commonly manifest in the abdominal area, but they can appear along any region of the sympathetic trunk, including the neck, hip and facial region.¹⁵ Affected children may exhibit weight loss, diarrhea, fever, as well as increased urination or trouble breathing as a consequence of tumor compression.¹⁶ The cancer most commonly metastasizes to the skin, liver, and bones (especially the orbital bones).¹⁷ Metastasis to the orbital bones causes periorbital ecchymosis, more commonly known as “raccoon

eyes” (figure 2).¹⁷ Some patients with neuroblastoma may also develop bluish lesions on the skin known as “blueberry muffin skin lesions.”¹⁶

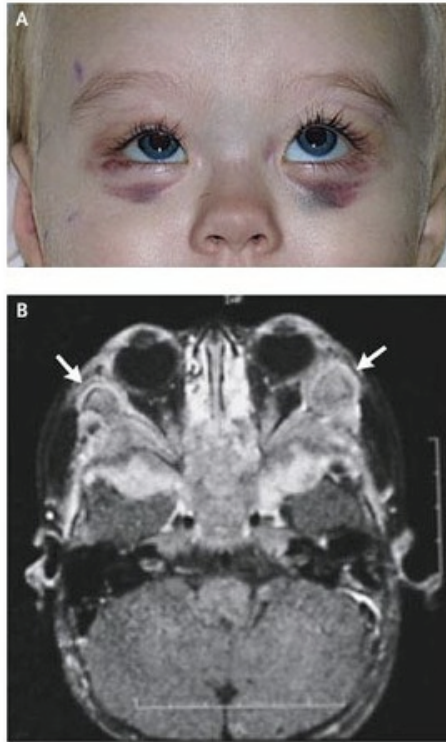


Figure 2. Periorbital Metastasis and Raccoon Eyes. Depiction of bilateral periorbital ecchymosis or “Raccoon Eyes” (A) and an MRI image (B) demonstrating metastasis of neuroblastoma to the skull. The tumor in (B) is associated with the zygomatic, sphenoid, and temporal bones. Image taken from Timmerman.¹⁸

Detection and Staging Methods

Routine screening for neuroblastoma in all children is not recommended, as it may lead to over-diagnosis and unnecessary treatment of tumors that would oftentimes mature and resolve on their own.¹⁹ Over-diagnosis and treatment can

lead to further complications and co-morbidities. When infants are suspected of having NB, urine is collected and tested for vanillylmandelic acid and homovanillic acid levels¹⁹. Both of these substances are metabolites of norepinephrine, epinephrine, and dopamine, which can be secreted by neuroblastoma tumors.²⁰ The urine screening method has a diagnostic sensitivity of 82%.²¹

Once NB is confirmed, it is essential to perform appropriate staging to understand the extent of disease. NB is staged using the International Neuroblastoma Staging System (INSS) that was originally proposed in 1988 and has been modified based on new findings as seen on Table 1.²² The INSS focuses on the primary tumor and the extent to which the disease has spread. About 75% of children diagnosed after the age of one are in stages 3 or 4, while infants younger than 1 year tend to be at the lower stages of 1,2, and 4s.¹⁵ Tumors that are categorized as stage 2B have been found to be the most aggressive.²³ NB may regress completely in infants, especially those in the 4s stage, even without the need of intense therapy.²³

Table 1. International Neuroblastoma Staging System. Risk increases with stage progression, excluding stage 4S which may regress spontaneously. Taken from Monclair et al.²²

Stage	Description
1	Tumor is localized to the area of origin and can be completely removed through surgery. Bilateral lymph nodes negative microscopically.
2A	Localized tumor and incomplete gross excision. Bilateral lymph nodes negative microscopically.
2B	Localized tumor with or without complete excision. Ipsilateral, non-adherent lymph nodes are positive, contralateral lymph nodes are negative.
3	Unresectable tumor infiltrates across midline, with or without proximal lymph node involvement; or localized tumor with contralateral regional lymph node positive.
4	Tumor spread to distant lymph nodes, skin, bone marrow, bone, liver, or other organs.
4S	Localized primary tumor as previously described in 1, 2A, 2B. Metastasis only to skin, liver, and/or bone marrow. Stage is limited to infants younger than 1 year.

It is equally important to determine the stage of the disease on a cellular level. Shimada et al proposed an international Neuroblastoma Pathology Classification based on age at the time of diagnosis, degree of cellular differentiation, nuclear morphology, and the organization of stromal tissue in the primary tumor.²⁴ Histological studies have noted that NB is associated with two major types of cells: one type is the meroblastic cells of neural crest lineage and the other consists of non-neuronal cells, including Schwann cells.²⁵ Schwann cells are part of the peripheral nervous system and are a type of support cell that produce the myelin sheaths that cover neuronal axons. When noting the stromal conditions of the samples, Shimada et al assigned them to two different

categories, stroma-poor and stroma-rich groups. Stroma-poor cases were characterized as being composed of mostly neuroblastic cells, while stroma-rich cases were those that contained higher amounts of Schwann cells or other supporting elements.

A level of differentiation was also assigned to each category. Stroma-poor cases were graded as being either “non-differentiated” or “differentiating”. The “non-differentiated” label indicates that less than 5% of the neuroblasts were differentiating. The “differentiating” label indicates that at least 5% of the cells were differentiated or of higher maturity. Stroma-rich cases were graded to be either “well differentiated” or “intermixed”. Stroma-rich tumors were considered to be ganglioneuroblastoma rather than the classical neuroblastoma, as they were typically composed of more differentiated cells, whereas the stroma-poor groups were identified as classical neuroblastoma or diffuse ganglioneuroblastoma. A second study performed by Shimada et al confirmed that the younger the child at the onset of disease and the more differentiated the tumor cells, the more favorable the prognosis.²⁶

A comprehensive risk assessment is typically used to estimate the survival rate for patients. An international neuroblastoma risk group (INRG) classification system was designed as a way to establish a consensus on risk stratification.² The INRG includes four stages, L1, L2, M, and MS. L1 classification is assigned to localized tumors that do not involve vital structures and are confined to one compartment of the body. L2 classification is given to loco-regional tumors with

one or more image defined risk factor. M is defined by distant metastatic disease except for MS. Finally, MS is defined by metastatic disease in a child under 18 months of age with metastasis limited to skin, liver, and/or bone marrow. A summary of classification stages and relative histologic categories, ploidy, MYCN status, and risk level is shown in table 2.

Table 2. Pre-treatment Risk Analysis of Neuroblastoma. Risk is based on stage, age of patient at diagnosis, histologic classification, tumor differentiation, MYCN amplification, and chromosomal aberrations (taken from Cohn et al).²

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 GN maturing or GNB intermixed		NA			B Very low
				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				NA	No		C Very low
	< 18				Yes		Q High
					Amp		

ORIGIN OF NEUROBLASTOMA

Neuroblastoma may commonly affect the adrenal glands. The adrenal glands are located superiorly to the kidneys and are comprised of three layers: the capsule, the cortex, and the medulla as seen on figure 3. The medulla houses chromaffin cells with the ability to produce the stress hormones epinephrine, norepinephrine, and dopamine when stimulated by sympathetic neurons. Once released, the hormones cause the mobilization of glucose stores and increase both heart rate and blood pressure.

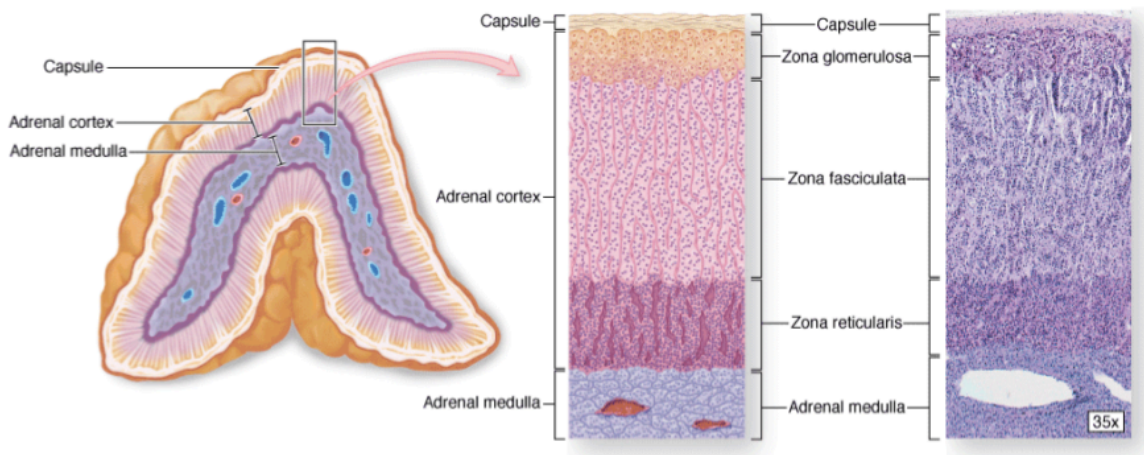


Figure 3. Layers of the Adrenal Gland. Layers include the adrenal capsule, cortex, and medulla. Many neuroblastomas are associated with chromaffin cells of the adrenal medulla. Taken from Junqueira's Basic Histology by Mescher.²⁷

Neuroblastoma has been shown to affect differentiating chromaffin cells, which are of neural crest origin. Cells in the adrenal gland cortex are typically not involved in NB pathogenesis and are not derived from neural crest cells. It is,

therefore, important to understand embryogenesis and the development of neural crest cells in order to decipher the pathogenesis of NB.

The first four weeks of embryogenesis involve the formation of three functional germ layers that will eventually give rise to the embryo's skin, nervous system, and organs. These layers are the ectoderm, mesoderm, and endoderm. The adrenal medulla is formed from the ectoderm layer while the adrenal cortex is derived from the mesoderm. Both the mesoderm and endoderm play a major role in the development of muscles, organs, and the epithelium of the gut and respiratory systems. The ectoderm layer will give rise to the nervous system and the skin. Some cells of the ectoderm layer will differentiate to form a thickened area known as the neural plate. The neural plate, which eventually gives rise to the central nervous system, folds in to form the neural tube. The crest of the fold is commonly known as the neural crest and once the folding process is over, the cells in this location will disassociate to form neural crest cells. The neural tube then sinks below the ectoderm and the ectoderm fuses over it (figure 4) to later form the skin.

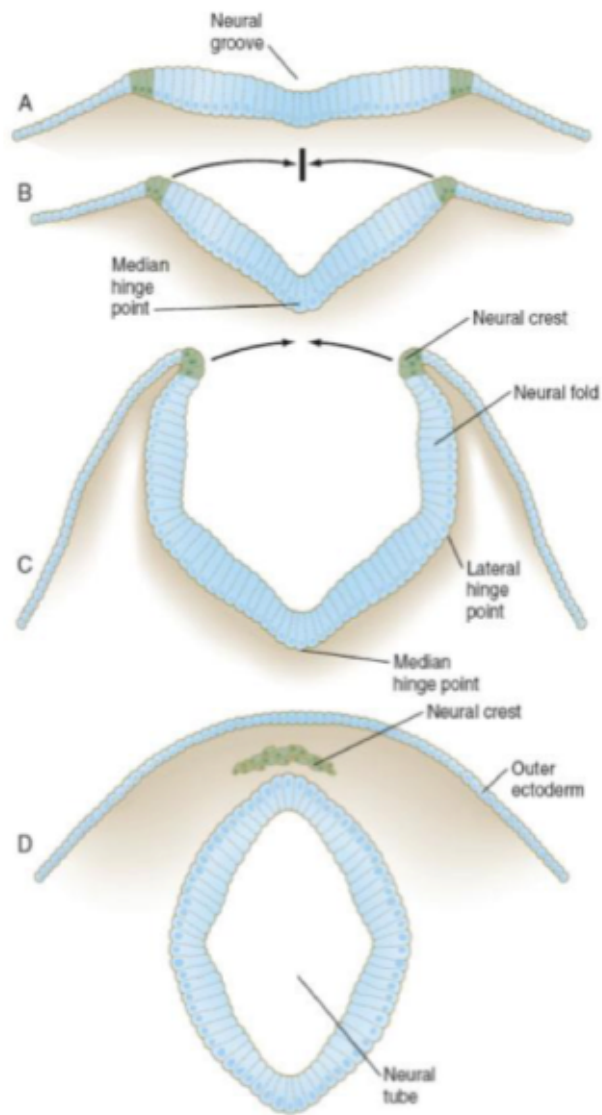


Figure 4. Formation of Neural Tube from Ectoderm. A portion of ectoderm thickens (A) and folds (B,C) to form the neural tube (D). Neural crest cells, which originate from the crest of the fold, detach once the neural tube closes. Figure taken from Schoenwolf et al.²⁸

As the embryo continues to develop, neural crest cells delaminate from the neural tube and migrate from the ectoderm layer to the mesenchyme, a process known as epithelial-mesenchymal transition (EMT). Neural crest cells, which have migratory and pluripotent capabilities, travel to other areas of the embryo to give rise to a variety of structures. They have the potential to differentiate into peripheral neurons, smooth muscle cells, chondroblasts, osteoblasts, and chromaffin cells depending on the axial level of the body to which they migrate to.¹³ The process of induction and migration of neural crest cells is regulated by a complex network of transcription factors, also known as neural crest specifiers (i.e MYCN, c-myc, Id2, PHOX2, Sox9, Sox10, Twist, and Slug/Snail).²⁸ The signaling molecule bone morphogenic protein and the transmembrane receptor NOTCH appear to play a role in the regulation of these transcription factors.²⁹ In addition to allowing the cell to migrate, these specifiers can also assign a particular neural crest lineage and control differentiation.³⁰ Alterations in the functioning of neural crest specifiers may be involved in the upregulation of proliferation and the suppression of differentiation, leading to tumorigenesis and malignancy.²⁸ It is likely that the neoplastic event that causes neural crest cells to become carcinogenic occurs sometime after EMT but before complete differentiation.³¹ Interestingly, neuroblastoma can develop from neural crest cells in the sympathoadrenal lineage and not from other lineages.³²

DISEASE MECHANISMS

Understanding the mechanisms by which neuroblastoma develops is incredibly important, as it can help scientists develop therapies that specifically target the disease pathway. Although these mechanisms have not been completely deciphered, some of the key players of this process have been identified and involve several functional and genetic alterations.

Tyrosine Kinase Receptors

ALK belongs to the tyrosine kinase receptor family, which includes other members like the epidermal growth factor receptor (EGFR/HER-1) that is associated with breast cancer. These receptors play a vital role in the cancerous transformation of the cell, as they involve the regulation of cellular growth, proliferation, and differentiation. ALK mutations have been found to be the main cause of familial neuroblastoma,³³ although activating somatic mutations have also been found in sporadic NB.³⁴ A study by George et al, found that some neuroblastomas exhibited ALK amplification while others had mutated ALK kinase domains.³⁴ The study also found 5 previously unidentified ALK mutations of which two were germline mutations and two were somatic. The most common mutation appeared to be F1174L, which involves the substitution of an amino acid at position 1174 from a phenylalanine (F) to a leucine (L). This ALK mutation is also found in some types of lung cancer and has been thought to cause an

acquired resistance to Crizotinib, a drug that functions as a tyrosine kinase inhibitor.³⁵ Aside from F1174L, approximately two dozen ALK mutations have been identified in total.³⁶

ALK contains 1620 amino acids and like other tyrosine kinase receptors, it has an intracellular catalytic domain, a transmembrane domain, and an extracellular ligand-binding domain; ligands include pleiotrophin and midkine, 2 homologous growth factors that are expressed in the embryo but decrease at birth.³⁷ After ligands bind to the extracellular domain, the receptors dimerize and cross phosphorylate. Mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), and phosphoinositide phospholipase C γ (PLC γ) pathways can be activated by phosphorylated ALK as seen on figure 5. These pathways lead to cellular proliferation, migration, differentiation, and the activation of survival mechanisms.³⁸

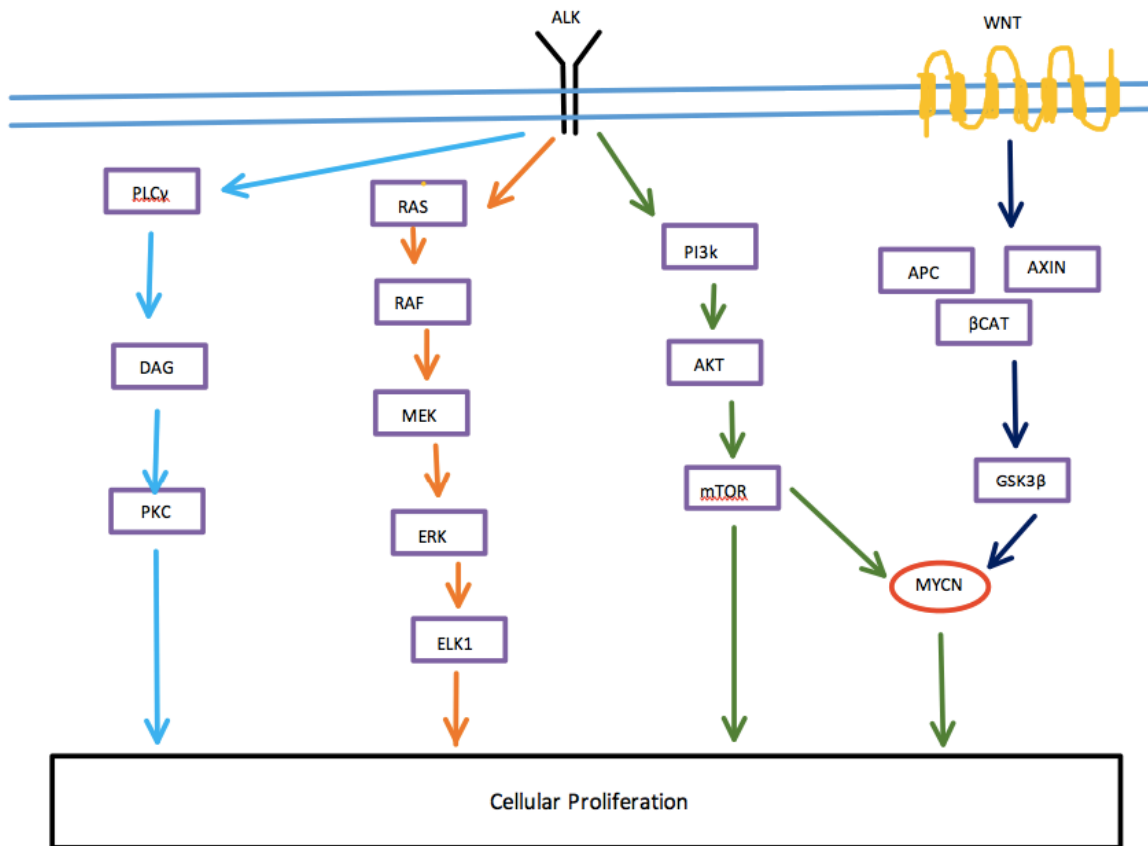


Figure 5. Proposed ALK and WNT Mechanisms. ALK can activate RAS, PI3K, and PLC γ pathways to promote tumorigenesis. WNT acts through the canonical, β -catenin dependent pathway to promote tumorigenesis. Both pathways may interact with MYCN downstream. Figure adapted from findings by Holla et al and Johnsen et al.^{38,39}

PI3K is a protein kinase that, in its inactive mode, has an adapter protein bound to its regulatory subunit. Once activated by ALK, PI3K will convert phosphatidylinositol (3,4)-bisphosphate (PIP₂), a minor phospholipid component of the cellular membrane, to phosphatidylinositol (3,4,5)-triphosphate (PIP₃).⁴⁰ An increase in the concentration of PIP₃ will activate AKT via the removal of a regulatory domain that blocks the AKT kinase domain. AKT will then phosphorylate and activate a protein known as mammalian target of rapamycin

complex 1 (mTORC1), leading to an increase in proliferation and protein synthesis through its actions on translational factors and ribosomal kinases.⁴¹

In another pathway, activated ALK can subsequently lead to the activation of PLC γ . PLC γ can interact with PIP₂, hydrolyzing it into inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 increases the concentration of free Ca²⁺ in the cytosol and activates protein kinase C (PKC), which plays a role in cellular proliferation and differentiation.⁴² The role of PKC in the progression of neuroblastoma has been confirmed in in-vitro studies where the addition of PKC inhibitors such as H7 and Calphostin C resulted in decreased proliferation of neuroblastoma cells.⁴³

In the MAPK pathway, adaptor protein Grb2 binds to the phosphotyrosine tail of the receptor and to the guanine nucleotide exchange factor (GEF) SOS, which then activates a protein known as RAS by exchanging guanosine diphosphate (GDP) to guanosine triphosphate (GTP).⁴⁴ Activated RAS will recruit RAF, a serine protein kinase, to the cellular membrane. RAF can then activate protein kinase MEK, and MEK can activate another protein kinase, ERK. ERK acts on transcription factors in the nucleus to upregulate proliferation.⁴⁴ The MAPK signaling cascade in a normal cell is terminated and regulated in several ways. One of these is the spontaneous hydrolysis of GTP to GDP which will deactivate RAS. Other ways include the internalization of the ALK receptor and the deactivation of components by phosphatases in the cell. There has been evidence of RAS-MAPK activation in neuroblastoma tumor progression.⁴⁵ In fact,

molecular studies have found that Neuroblastoma RAS (NRAS) is significantly mutated in a small number of high risk patients.⁴⁶ Increased RAS-MAPK activation increases the expression of MAPK feedback inhibitors like dual specificity phosphatases (DUSPs) and sprouty proteins (SPRYs).⁴⁷

There are several other tyrosine kinase receptors that have been linked to NB, specifically TrkA, TrkB, and TrkC. Expression of TrkA, which is activated by the ligand nerve growth factor (NGF), is associated with better prognosis and is inversely correlated to MYCN amplification in NB.^{11,48} However, TrkB expression is associated with unfavorable prognosis, has positive correlation with MYCN amplification, and is present in approximately more than half of high-risk neuroblastoma cases.⁴⁹ Brain derived neurotrophic factor (BDNF) is a ligand that binds to TrkB receptors. Studies have reported that the activation of this receptor with BDNF can lead to drug-resistance, angiogenesis, and increased survival of tumor cells.^{50,51} Similar to TrkA, expression of TrkC appears to be favorable, is more common in low-risk disease, and has an inverse correlation with MYCN amplification.⁵²

PTPN11

About 3.4% of NB cases, particularly higher risk stages, contain mutations in the tyrosine-protein phosphatase non-receptor type 11 gene (PTPN11).⁴⁶ PTPN11 codes for the protein SHP-2, which is involved in the activation of the RAS-ERK and PI3K-AKT pathways.⁵³ SHP-2 is also involved in EMT and the

modulation of focal adhesion kinases that keep the cell adhered to components in the extra cellular matrix.⁵³ In fact, research shows that SHP-2 activation can promote metastasis and invasion of oral cancer.⁵⁴ The sequencing of PTPN11 from 13 different human cancers including neuroblastoma revealed 11 missense mutations, 5 of which caused the activation of SHP-2.⁵³ A missense mutation, or point mutation, involves the substitution of a single nucleotide base and results in a codon that codes for a different amino acid. A change in an amino acid can lead to a change in protein structure and function. Synthetic inhibitors of SHP-2 have demonstrated varying degrees of success when tested on different types of cancer. SHP099 and salicylic acid derivatives have been shown to inhibit cell proliferation and migration by inhibiting SHP-2, but this success seems to occur only when cells have intact, mutation-free, RAS-ERK pathways.^{55,56}

MYCN

MYCN amplification is considered to be the most important prognostic marker for high-risk neuroblastoma and is present in about 20% of cases.³² MYCN is structurally related to another neural crest specifier c-myc, as two of their domains show a 78% and 83% similarity.⁵⁷ Although both normal neural crest cells and neuroblastoma cells express the transcription factor MYCN, a study performed on mice demonstrated that MYCN levels progressively decreased in normal cells during development, but became consistently elevated in tumor cells.³¹ The over-expression of MYCN, with no other contributing factors

can cause neuroblastoma in mouse models.³¹ A study on the extent MYCN amplification in NB revealed that MYCN DNA could be amplified 20 to 140 fold in NB cells and that this amplification was more commonly seen in high risk patients with stage 3 or 4 cancer than those with stages 1 and 2.⁵⁸ MYCN plays a significant role in high risk neuroblastoma by promoting protein synthesis, proliferation, angiogenesis, metastasis, and immune system evasion through complex pathways.⁹ A summary of some of these cellular interactions can be seen in figure 6.

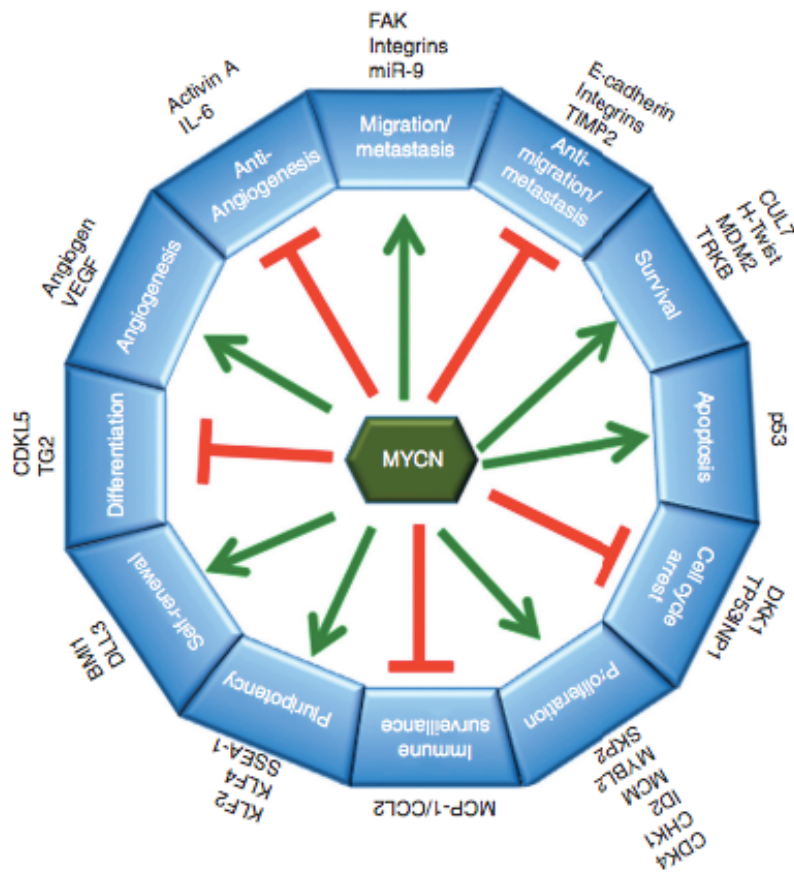


Figure 6. Role of MYCN in Neuroblastoma. MYCN amplification can promote malignancy in a variety of ways.

MYCN can inhibit cellular differentiation in numerous ways. Research has shown that MYCN can suppress the activity of alpha estrogen receptors (ER α) and NGF, which are both important for cellular differentiation.⁵⁹ As noted on the Neuroblastoma Pathology Classification System, a less differentiated tumor is typically associated with a more malignant disease and unfavorable prognosis. MYCN can also block cellular differentiation by down-regulating CDKL5; although the exact actions of CDKL5 are not clear, an increase in this protein promotes cell cycle arrest and differentiation.⁶⁰ In addition, knocking out MYCN expression along with other myc genes in neuroblastoma cell lines resulted in severely impaired pluripotency, self-renewal properties, and viability.⁶¹

Metastasis is yet another major concern in NB, as it has been reported to occur in approximately 50% of all cases.⁶² A research study on the regulation of metastasis found that the micro-RNA miR-9 was up-regulated in cancer cells and that increased levels correlated with increased metastasis.⁶³ Typically, micro-RNAs (miRNAs) are able to decrease post-transcriptional expression of specific proteins by binding to the protein's own messenger RNA (mRNA) and destabilizing it. Ma et al found that miR-9 targeted the mRNA of the cell-to-cell adhesion molecule E-cadherin (CDH1), thereby increasing motility and promoting metastasis.⁶³ Previous studies have shown that decreased E-cadherin levels can lead to increased expression of vascular endothelial growth factor (VEGF) via β -catenin dependent pathways.⁶⁴ Ma et al ultimately determined that miR-9 was activated by MYCN and that levels of miR-9 correlated with MYCN

amplification.⁶³ This is a mechanism that still needs to be studied specifically in neuroblastoma cell lines, as the researchers used breast cancer cells for their analyses.

Much of the research being conducted demonstrates that there is an intricate relationship between MYCN amplification and the various ALK pathways. One major finding was that ALK signaling promoted the transcription of MYCN in both neuroblastoma and wild-type cells.⁶⁵ This suggests that patients whose tumor cells express activating mutations of the ALK gene may also experience significant MYCN amplification and exhibit an aggressive form of cancer. Indeed, George et al, found that in a sample of 94 tumors with MYCN amplification, 15% also exhibited ALK amplification.³⁴ The study also found that ALK amplification could not be detected in the 51 tumors that did not demonstrate MYCN amplification.³⁴

Recent studies indicate that there is more than one mechanism by which ALK can promote MYCN expression. The PI3K-AKT pathway in particular has drawn much attention, as it appears to be the pathway most heavily associated with MYCN amplification and viability. A study performed by Berry et al on a mouse model of neuroblastoma proposed that the ALK-PI3K-AKT pathway increases the oncogenic effects of MYCN protein by promoting its stabilization.⁶⁶ They demonstrated that P13K inactivates glycogen synthase kinase 3 β (GSK3 β), which can phosphorylate and deactivate MYCN; phosphorylated MYCN then becomes a target for ubiquitination and degradation.⁶⁶ GSK3 β essentially acts as

an inhibitor of MYCN, but the continued activation of the ALK-PI3K pathway down-regulates this inhibitor and contributes to MYCN viability in the cell. Furthermore, a 2018 study by Claeys et al. found that PI3K signaling positively regulates MYCN by suppressing the expression of HMG-box transcription factor 1 (HBP1).⁶⁷ HBP1 has been shown to act as a negative regulator of MYCN.⁶⁸ Claeys et al also reported that MYCN itself could indirectly repress HBP1 levels, as it up-regulates a miRNA cluster known as miR-17~92, which targets HBP1 mRNA.⁶⁷

WNT

WNT signaling functions through various frizzled (FZ) type receptors and regulates important cellular functions such as differentiation, migration, and cell polarity (figure 5).⁶⁹ The structure of FZ receptors is similar to that of G-protein coupled receptors, as they also contain seven transmembrane domains. WNT glycoproteins are bound to the N terminal of FZ receptors and, once activated, can transduce a signal involving numerous downstream proteins. There are three main WNT signaling pathways, one being β -catenin dependent (also referred to as canonical) and two that are β -catenin independent, which include the planar-cell-polarity (PCP) and the WNT-Ca²⁺ pathways. Comparatively, more research has been done on the role of tyrosine kinase pathways and MYCN in neuroblastoma than that of WNT signaling pathways.

The different WNT signaling pathways may play distinct roles in NB pathogenesis. For instance, high risk human NB samples have been reported to have decreased levels of WNT5A, which is considered to be a part of the β -catenin independent pathway.⁷⁰ On the other hand, Dyberg et al confirmed that low-risk neuroblastoma was associated with decreased activity of the β -catenin dependent pathway.⁷¹ Dyberg et al also found that low risk neuroblastoma had increased levels of the PCP pathway components VANGL2 and PRICKLE1.⁷¹ It has been previously demonstrated that PRICKLE1 is an inhibitor of the β -catenin canonical pathway.⁷² The findings of these studies ultimately support the idea that the β -catenin independent pathways correlate with a favorable prognosis, while also proposing that the β -catenin dependent pathways may potentiate the disease. Although the role of the β -catenin dependent pathway is still not completely understood, research indicates that one of the receptors through which it operates (FZD1) may mediate the expression of a multidrug-resistant protein (MDR1) that causes treatment resistance in tumor cells.⁷³ It is unknown whether or not MYCN interacts with WNT signaling as it does with ALK.

ARID1A and ARID1B

A study by Sausen et al sequenced the genome of 71 NB tumors and found that 11% of the sample contained chromosomal deletions and alterations of ARID1A and ARID1B genes.⁷⁴ ARID1A and ARID1B code for SWI/SNF proteins involved in chromatin remodeling, a process that alters how tightly DNA

is packaged and regulates gene expression.^{75,76} SWI/SNF proteins may also act as tumor suppressors and are involved in the regulation of cellular growth, division, maturation, DNA replication, and repair.^{75,76} A recent study by Kelso et al showed that the deletion of ARID1A and ARID1B was highly associated with changes in gene expression, particularly genes that coded for signaling intermediates in cellular growth and adhesion.⁷⁷ The exact effects of ARID1A and ARID1B deletions in NB are not clear, but studies on liver cancer showed that ARID1A deletions promote progression and metastasis.^{78,79}

ATRX

ATRX loss of function mutations were found in 9.6% of patients in a study of 240 high-risk neuroblastoma cases, particularly in children over 18 months of age.⁴⁶ ATRX mutations appear to be especially prominent in adolescent and adult patients.³ ATRX is an RNA helicase and the loss of its function leads to the lengthening of telomeres in tumor cells, although this effect seems to occur mainly in NB tumors that do not exhibit MYCN amplification.^{80,3} Telomeres are proteins that protect the ends of chromosomes from deterioration. The length of telomere segments usually decreases as cells continue to replicate. Typically, when telomeres become too short, cells lose their ability to replicate and begin to senesce. Thus, an increase in telomere length may increase the longevity of tumor cells as well as their replicative capability. Nonetheless, more research is

needed to determine the role of ATRX mutations in neuroblastoma and how they can lead to telomere lengthening.

Neuritogenesis Genes

Neuritogenesis is a process by which neurites (axons or dendrites) form. It is a complex signaling process that involves the GTPases Rho and Rac.⁸¹ Molenaar et al discovered that several proteins and regulators in the neuritogenesis pathway had undergone mutations in neuroblastoma, particularly in aggressive stage 3 or 4 tumors.⁸² Some of the mutated genes (PTPRD, ODZ2, ODZ3, CSMD1) are associated with neurite formation, while others (TIAM1, DLC1, ARHGAP10) function as Rac/Rho regulators.⁸² A 2017 study by Dyberg et al suggested that the mutations may involve the activation of downstream Rho associated kinases (ROCKs).⁸³ In fact, the study demonstrated that increased expression of ROCK2 is linked to poor outcomes in NB and the inhibition of it can suppress tumor growth and promote MYCN degradation.⁸⁴

CHROMOSOMAL INSTABILITY

In addition to MYCN and ALK amplifications, there are other chromosomal abnormalities associated with NB, these include hyperploidy, chromothripsis, the deletion of chromosome arms 1p, 3p, 4p, and 11q, as well as the gain of

chromosome arm 17q.⁸⁵ About one-third of tumors are diploid, while two-thirds are hyperploid.⁸⁶ A human cell normally contains 23 pairs of chromosomes. A cell is considered hyperploid when it contains one or more chromosomes in excess. Tumor ploidy is an important prognostic marker in neuroblastoma, especially because infants with hyperploid tumors have increased survival rates.^{2,14} A study performed by Buckley et al. summarized the chromosomal aberrations that could be found in the neuroblastoma cell genome (figure 7).⁸⁷ The researchers also calculated the frequency of MYCN and ALK amplifications, both of which appear on chromosome 2 (location 2p24.1 for MYCN and 2p23.1 for ALK).⁸⁷ Figure 8 depicts the risk level associated with these chromosomal alterations.

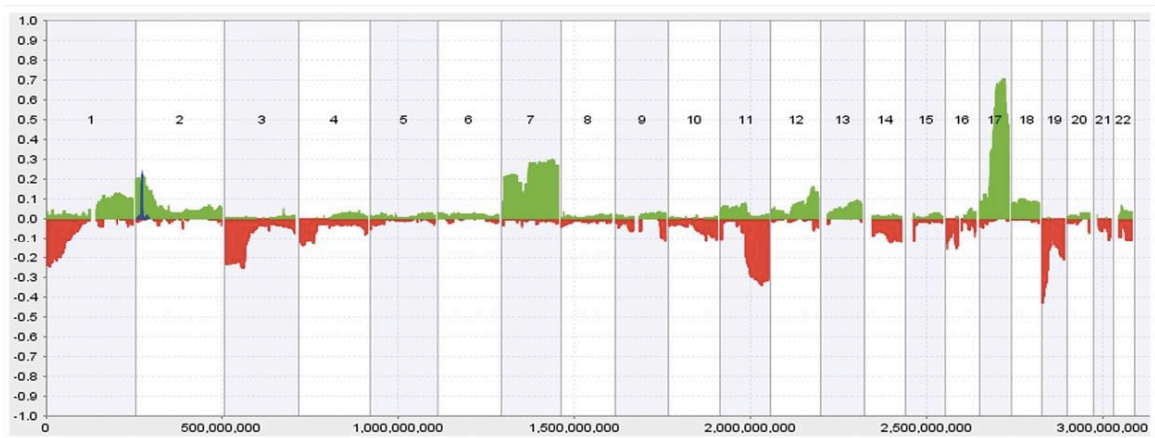


Figure 7. Frequency of Segmental Chromosome Alterations. There are multiple additions (green) or deletions (red) of chromosome segments in neuroblastoma. Frequency of MYCN and ALK amplification is depicted by the blue line on chromosome 2. Figure taken from Buckley et al.⁸⁷

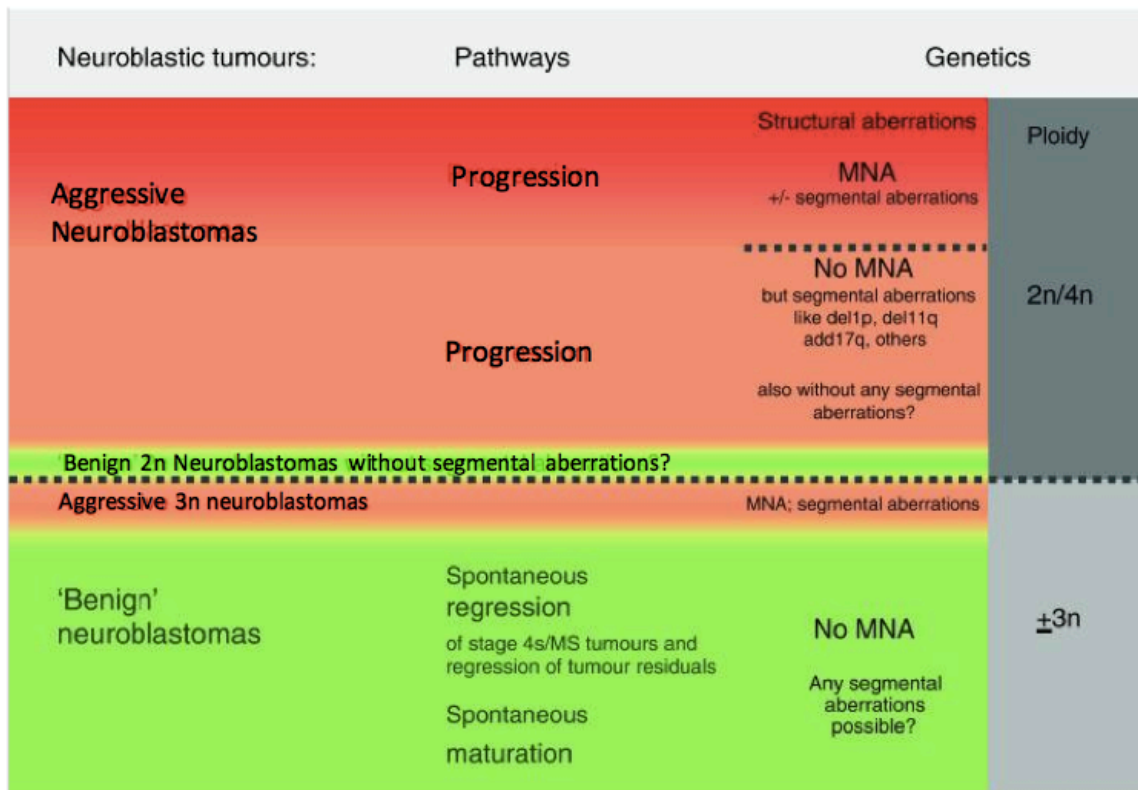


Figure 8. Associated Risk Level for Tumors with Differing MYCN Status, Ploidy, and Chromosome Alterations. MNA represents MYCN amplification. Segmental or structural aberrations appear to play a role in disease aggressiveness but MYCN amplification remains the most important predictor. Taken from Ambros et al.⁸⁸

Loss of 11q Alleles

The loss of 11q can be relatively common in neuroblastoma, occurring in about 44% of sporadic cases and associated with increased malignancy.⁸⁹ Interestingly, loss of 11q is inversely correlated with MYCN amplification.⁸⁷ Still, it is essential to find how the loss of alleles in this location can lead to tumorigenesis. Several of the 11q genes that may play a role in pathogenesis have already been identified and are currently being studied, including H2AX,

CADM1, ATM, PHOX2A, and SDHD.⁹⁰ H2AX, for instance, codes for a histone protein. Histone proteins play an important role in the packaging of DNA and are involved in processes like transcription, replication, and the maintenance of chromosome stability.⁹¹ The loss of one H2AX allele can lead to chromosomal instability and increased risk of developing cancer.⁹² Furthermore, decreased expression of CADM1, cell adhesion molecule 1, has also been associated with a poor prognosis in several types of cancers, particularly those that are invasive and undergo metastasis.^{93,94} When neuroblastoma cells were made to over-express CADM1, a decrease in proliferation was observed in-vitro.⁹⁵ Another study focused on the role of ataxia-telangiectasia mutated (ATM) gene and found that haploinsufficiency of the ATM gene, which functions as a cell cycle checkpoint kinase and tumor suppressor, is also linked to lower cancer survival rates.⁹⁶ The gene PHOX2A has been identified as a transcription factor that promotes noradrenergic neuronal differentiation during embryological development.⁹⁰ It is regulated by a homologous molecule, PHOX2B (located on chromosome 4) and known for its association with familial neuroblastoma as well as its role as a neural crest developmental regulator.⁹⁰ Microarray analysis revealed that there was decreased expression of PHOX2A in unfavorable tumor cells, though it is still not understood how lower levels of this transcription factor lead to pathogenesis.⁹⁷

Finally, the tumor suppressor gene SDHD (located on 11q23) was also considered a potential candidate for neuroblastoma tumorigenesis because a

mutation of this gene had been linked to the onset of paraganglioma.⁹⁸ A study performed by De Preeter et al, confirmed lower SDHD expression in cells with loss of the 11q allele.⁹⁹ However, no evidence for SDHD involvement in NB pathogenesis was found, as they did not see signs of increased methylation or significant mutations of the gene in neuroblastoma cell lines.⁹⁹ Thus, the researchers suggested that a haploinsufficient mechanism be considered for possible cause of disease.

Gain of 17q Alleles

One of the most common genetic aberrations associated with advanced or aggressive neuroblastoma is the gain of 17q, which occurs in approximately 70% of cases and involves translocations of regions 1p and 11p, among others.⁸⁵ The 17q region that is gained contains about 18 genes, some of which are part of the ABCA ATP-binding transporter family that is known for causing drug resistance in tumors.^{87,100} Somatostatin receptor 2 (SSTR2) and the neural crest specifier SOX9 are also located in the 17q region.⁸⁷ However, SSTR expression does not appear to be involved in tumorigenesis. Some studies have demonstrated that expressions of SSTR-1, 2, 3, and 4 were significantly higher in NB of favorable prognosis than in the unfavorable group.^{101,102} Somatostatin, the ligand for SSTR, usually has an inhibitory effect on hormonal pathways, secretion, and cellular growth.¹⁰³ The contradictory outcomes for 17q addition and SSTR

expression shows the incredible complexity of neuroblastoma tumors and highlights the need for further research.

The transcription factor and neural crest specifier SOX9 has a role in maintaining stem cell phenotype, restricting cell lineage,¹⁰⁴ and contributes towards epithelial mesenchymal transition as well as delamination.²⁹ Like SSTR, the role that SOX9 may play in NB tumorigenesis is unclear, as it has exhibited contradicting properties in different types of cancer. Increased expression of SOX9 in colorectal cancer promotes proliferation.¹⁰⁴ In contrast, SOX9 appears to behave as a tumor suppressor in some types of melanoma,¹⁰⁴ a cancer that involves another neural crest derived cell, the melanocyte.

There is disagreement in the scientific community regarding the use of a gain in 17q as a prognostic factor for overall survival (OS) and event-free survival (EFS). While several studies indicate that gain of 17q is an independent predictor of a poor prognosis,^{105,106,107} other studies stated that 17q gain was not predictive of outcome in the absence of MYCN amplification or 11q deletion.^{86,108} Nonetheless, almost all tumors that show MYCN amplification also exhibit 17q gain,¹⁰⁵ perhaps suggesting a connection between the two prognostic factors. With this in mind, Godfried et al decided to study the downstream pathway of MYCN and determine if any genes on 17q were involved in the signal transduction.¹⁰⁹ His group identified the 17q genes nm23-H1 and nm23-H2 as being potential players in the MYCN pathway, as they were expressed 6 to 10 times more in MYCN+ neuroblastoma cells than those cells that did not have

MYCN amplification. The study hypothesized that the nm23 genes played a role in the malignancy of unfavorable neuroblastoma, though more extensive research is needed to understand the complete mechanism.

Chromothripsis

The genome sequencing study by Molenaar et al revealed that NB tumors with poor prognoses may exhibit chromothripsis,⁸² a phenomenon discovered in 2011¹¹⁰ characterized by a massive genomic rearrangement occurring in a single event. Chromothripsis appears to be associated with MYCN amplification and the loss of heterozygosity of chromosome segment 1b.⁸² The mechanism by which chromothripsis occurs is not known, but studies suggest it is a result of localized shattering of DNA and double stranded breaks.¹¹¹ Molenaar et al suggested that chromothripsis may also occur from mutations in the fanconi signaling pathway, as the majority of NB cases that exhibited this phenomenon had inactivating mutations on fanconi-associated nuclease 1 (FAN1) and fanconi anemia complementation group M (FANCM).⁸² In addition to NB, evidence of chromothripsis has been found in several different cancers,¹¹² including, but not limited to, acute lymphoblastic leukemia,¹¹³ invasive bladder carcinoma,¹¹⁴ glioblastoma¹¹⁵ and osteosarcoma.¹¹⁰

REGRESSION MECHANISM

Neuroblastoma is a complex and heterogeneous disease, as it can show large variation in clinical behavior ranging from highly aggressive and malignant tumors to tumors that regress spontaneously without any kind of treatment and eventually turn into a benign form of ganglioneuroma. Understanding the mechanism of regression and spontaneous differentiation is just as important as understanding pathogenesis, since the mechanisms can potentially contribute to the development of new therapeutic drugs that will promote natural regression. Like many other mechanisms in neuroblastoma, the mechanism for spontaneous regression is not certain but several research studies have proposed pathways based on gathered evidence.^{116,117,118}

Much of the research on the process of regression has focused on stage 4s NB tumors because it is most commonly observed in this stage.²³ TrkA has been identified as being a key player in the differentiation mechanism as there is increased TrkA expression in tumors with favorable prognosis.^{48,11} A 1997 study by Nakagawara and Brodeur demonstrated that when cells obtained from stage 4s neuroblastomas were cultured and exposed to exogenous NGF (a TrkA ligand), the cells survived and were able to differentiate completely, thereby exhibiting a similarity to neuroblastomas that differentiated spontaneously into a benign, and more mature ganglioneuroma.¹⁰ The study also showed that a lack of exogenous NGF caused cell death, mimicking the tumor regression process

that is observed in some patients.¹⁰ Overall, these studies suggest that finding novel ways to up-regulate TrkA in NB tumor cells may be beneficial for patient survival.

Normally, apoptosis, a form of programmed cell death, is controlled by enzymes known as caspases.¹¹⁹ However, an electron microscope analysis of neuroblastoma regression showed that the cells were not dying via caspase regulated apoptosis, but rather via autophagy, another form of regulated cell death.¹²⁰ Interestingly, the expression of Ras, an oncogene that participates in MAPK signaling, has been associated with a favorable prognosis¹²¹ and can reportedly trigger cell death through a caspase-independent pathway.¹²² Kitanaka et al tested this theory specifically on neuroblastoma cell lines and confirmed that high levels of RAS were associated with the caspase-independent programmed death of cultured tumor cells.¹²³ This is certainly a surprising observation, as the RAS-MAPK signaling cascade appears to be involved in tumorigenesis.⁴⁵ More research is needed to understand the elusive role of RAS and its potential involvement in NB regression.

Several genetic factors may be linked to the spontaneous regression of neuroblastoma. Gene regulating factors such as promoter methylation as well as histone and chromatin modifications may be involved in the regression of tumors.⁸ Another genetic factor that has been suggested as a possible cause for spontaneous regression is a decrease in telomerase. Reportedly, stage 4s tumors exhibit lower levels of telomerase when compared to the more aggressive

types of neuroblastoma.^{124,125} A few factors are thought to play a role in the inhibition of regression, including MYCN amplification, the loss of chromosome segment 1p, and the gain of segment 17q.¹²⁶

An additional mechanism of regression may be the targeting of neuroblastoma cells by the body's immune system. Paraneoplastic opsoclonus is a condition characterized by the presence of anti-neuronal antibodies.¹²⁷ About 2% to 3% of children with neuroblastoma are also diagnosed with paraneoplastic opsoclonus¹²⁸ and typically have a high chance of survival.¹²⁹ Another important observation was that high-risk tumors had decreased expression of human leukocyte antigen (HLA) class I, while favorable 4s tumors expressed normal levels of it.^{8,130,131} HLA class I molecules work to aid the body's immune system in distinguishing endogenous proteins from those that may come from outside sources like an invading virus or bacteria. Taking into account these immunological studies, finding ways to up-regulate HLA class I molecules as well as anti-neuronal antibodies may prove to be a valid method of treating neuroblastoma.

CURRENT TREATMENT AND OUTCOMES

Current Treatment

Patients diagnosed with NB receive various forms of treatment ranging from resection of the tumor, radiation, chemotherapy, Iodine 131-MIBG therapy,

and targeted therapy such as Trk inhibitors.⁵ Treatment varies depending on the stage and risk of the cancer, with children in low and intermediate risk groups which are comprised of non-MYCN tumors and 4S infants with or without metastasis, having generally good prognoses. Usually these patients are observed without implementing therapy or they may be offered low dose chemotherapy and resection.¹⁷

High risk patients typically receive a treatment plan with four distinct phases: induction chemotherapy, local control, consolidation, and maintenance.¹⁷ After induction chemotherapy, local control involves resection of the tumor along with radiation to prevent local recurrence of the cancer.¹⁷ The consolidation phase aims to remove any remaining disease. It typically involves chemotherapy with autologous bone marrow transplantation, in which the patient's own blood forming stem cells are drawn, frozen, stored, and returned to the patient when they have finished chemotherapy and radiation.¹⁷ In consolidation therapy, the patient may receive medications like retinoic acid, which has been shown to suppress tumor growth and promote differentiation in NB cell lines.^{132,133} Matthay et al. showed that autologous transplantation of bone marrow improved survival in children with high-risk NB.¹³⁴ The study also reported that administering retinoic acid after chemotherapy and transplantation reduced the risk of cancer recurrence. However, a more recent study indicates that administration of retinoic acid after stem cell transplantation is inefficient in the treatment of high risk

NB.¹³⁵ More research is needed to measure the efficacy of using retinoic acid as part of standard consolidation treatment.

Outcomes

Event free survival (EFS) and overall survival (OS) rates vary relative to the patient's risk group. A summary of survival rates based on risk classification is shown in figure 9. The National Cancer Institute defines EFS as the amount of time that a patient remains free of complications related to the cancer after treatment has ended.⁵ These complications can refer to the return of cancer or any of its associated symptoms or complications of treatment. OS rate refers to the percent of people in a study who are still alive after diagnosis or treatment of their disease.⁵ OS rates have vastly increased since the 1990s as research on neuroblastoma therapies and the understanding of disease mechanisms continues to improve (figure 10).¹³⁶

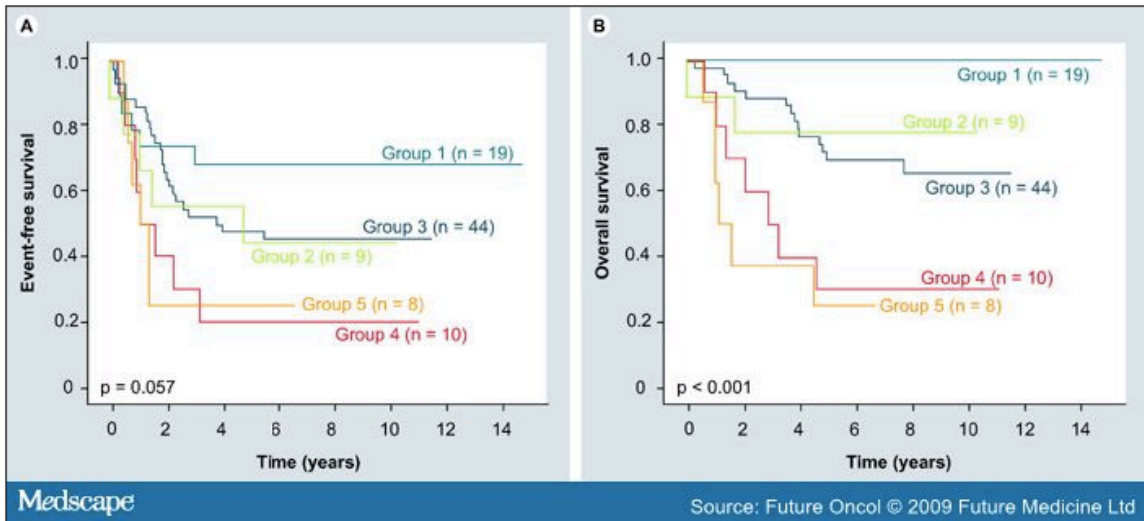


Figure 9. Overall and Event-free survival for Different Genomic Groups of Neuroblastoma. Figure is based on sample of 90 patients. Group 1 (n=19) refers to no chromosomal alterations or exclusive to numerical chromosomal alterations. Group 2 (n=9) is for structural chromosomal alterations, excluding MYCN amplification. Group 3 (n=44) is for both numerical and structural alterations without MYCN amplification. Group 4 (n=10) is for structural alterations and MYCN amplification. Finally, group 5 (n=8) represents structural and numerical alterations as well as MYCN amplification. Figure taken from Oberthuer et al.¹³⁷

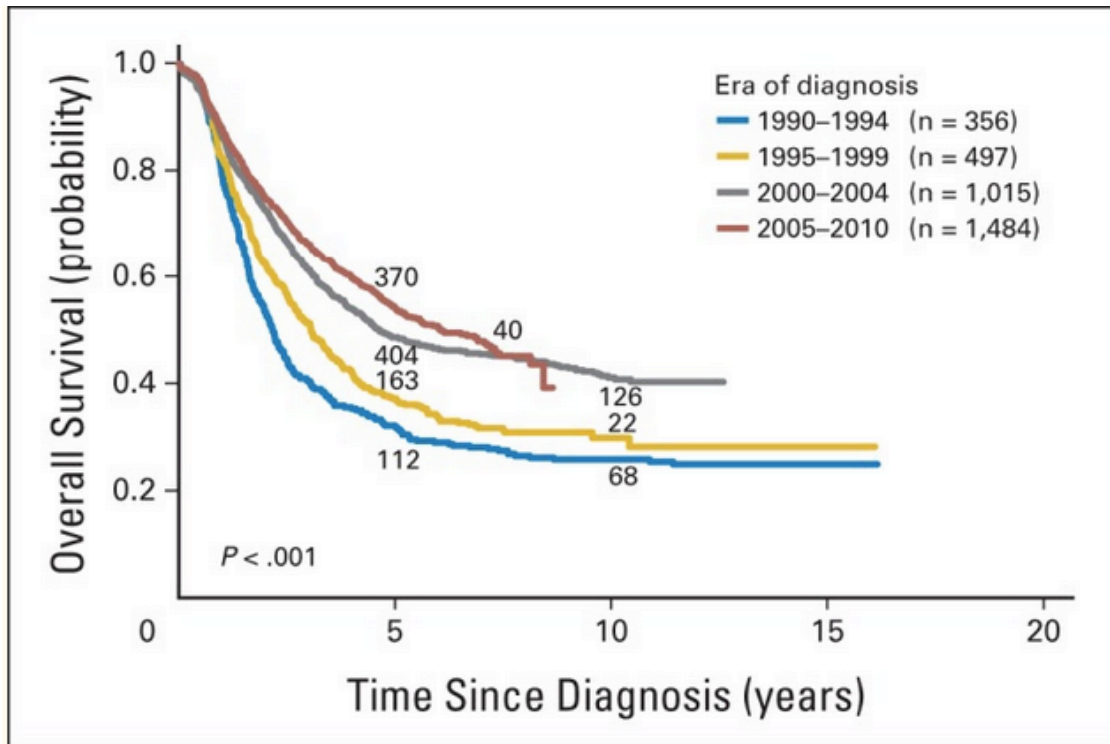


Figure 10. Improvement of Overall Survival of Neuroblastoma between 1990 and 2010. Survival probability has increased over the years as research continues to evolve. Taken from Pinto et al based on data collected by the Children’s Oncology Group.¹³⁷

TREATMENT RESEARCH

Immunotherapy

Immunotherapy is one of the main focuses of neuroblastoma research, attempting to find a way to enhance the body’s own immune system to target cancer cells. One of the therapies developed by Yu et al is a combination therapy with an anti-GD2 monoclonal antibody named ch14.18 (also known as

Dinutuximab), along with granulocyte-macrophage colony-stimulating-factor (GM-CSF), interleukin-2 (IL-2), and retinoic acid.¹³⁹ GD2 is a cell surface disialoganglioside that is almost exclusively expressed in NB tumor cells.^{138,139} Melanocytes and neurons also express GD2,¹⁴⁰ although the latter are protected by the blood brain barrier and may not be exposed to immunotherapy agents.¹⁴¹ The trial completed phase III in 2012 and showed a significant increase in EFS and OS. Patients receiving immunotherapy had an estimated EFS of 66±5% compared to 46±5% for standard therapy. Similarly, immunotherapy patients had an estimated OS of 86±4%, while patients in the standard therapy group had an OS of 75±5%. Anti-GD2 monoclonal antibodies have since become an essential component of high risk NB treatment.¹⁴² Nonetheless, the treatment has several side effects including abdominal pain, hypersensitivity reactions, and capillary leak syndrome.¹⁴³ Yu et al reported that one patient died from complications of capillary leak syndrome caused by an accidental overdose of IL-2.¹³⁹ Given the high dose toxicity of IL-2, a similar phase III clinical trial is being conducted comparing the outcomes of this combination therapy with and without IL-2 after an autologous stem cell transplant is performed on the patient.¹⁴⁴ Another currently active phase 1 trial is adding new genes to anti-GD2 white blood cells (GD2 T cells) in order to prolong their lifespan and increase their chances of killing NB tumor cells.¹⁴⁵ In general, anti-GD2 immunotherapies have proven to be highly effective at targeting NB cells while doing minimal damage to healthy tissue, unlike highly toxic chemotherapeutic agents.¹⁴⁶

Inhibition of MYCN

Efforts have been made to design a drug that can suppress or inhibit MYCN. The cell cycle regulators Aurora Kinase A (AURKA) and Aurora Kinase B (AURKB) have been shown to potentiate the effects of the MYCN oncogene. AURKA appears to play a role in the stabilization of MYCN,¹⁴⁷ while AURKB has been identified as one of its transcriptional targets.¹⁴⁸ Alisertib is a drug that has been designed to function as an AURKA inhibitor, but the drug showed low response rates when given to patients who had been diagnosed with solid tumors, including NB.^{149,150} Combining Alisertib with Irinotecan and Temozolomide produced a synergistic effect and an increased response rate of 31.8%.¹⁵¹ Irinotecan acts as an inhibitor of topoisomerase¹⁵², a protein needed for untangling DNA during replication, while Temozolomide is an alkylating agent that halts proliferation by adding an alkyl group to DNA and altering its structure.¹⁵³ Like other chemotherapies, several patients in this combination study experienced neutropenia,¹⁵¹ a condition characterized by abnormally low levels of white blood cells known as neutrophils.

Researchers have tried to identify inhibitors that can target both AURKA and AURKB with the hopes of producing more favorable treatment results. Two inhibitors in this category are Tozasertib and CCT137690.^{154,155} Tozasertib led to cell cycle inhibition and induction of apoptosis.¹⁵⁵ It has been shown, however, that expression of ABCB1 transporters confers resistance to Tozasertib but not to Alisertib.¹⁵⁵ ABCB1 transporters transfer a variety of substrates across the cell

membrane and are known to cause resistance in the treatment of several cancers, including breast and ovarian cancer.¹⁵⁶ In addition, pre-clinical trial testing of CCT137690 on MYCN amplified cell lines led to a decrease in proliferation and MYCN expression.¹⁵⁴ Side effects of Aurora kinase inhibitors include neutropenia, gastrointestinal toxicity, hypertension, and stomatitis.¹⁵⁷

Other researchers have studied the MYCN signaling pathway to identify and target its mediators. A histone chaperone called FAcilitates Chromatin Transcription (FACT) has been identified as one of these MYCN mediators and was found to be involved in a forward feedback loop that contributed to the increased expression of both MYCN and FACT itself.¹⁵⁸ FACT inhibition was achieved using Curaxin, or CBL0137, which worked cooperatively with chemotherapy agents by preventing the repair of damaged DNA caused by the exposure to the chemotherapy drugs.¹⁵⁸

Inhibition of ALK

The tyrosine kinase receptor ALK is being targeted by two inhibiting drugs, Crizotinib and Ceritinib, with Certinib being 20 times more powerful than the former.¹⁵⁹ Although the drugs showed positive results in patients with other types of cancers, they were unable to produce optimal results in neuroblastoma patients, where neuroblastoma tumors appeared to be resistant because of acquired mutations in the ALK gene.¹⁵⁹ Shen et al tested the effectiveness of a combination therapy consisting of Crizotinib and an inhibitor of histone

deacetylase 8 (HDAC8) that is lethal to both HDAC8 and ALK.¹⁶⁰ HDAC8 is also an indicator of unfavorable prognosis in patients with neuroblastoma and its inhibition has been shown to promote differentiation.¹⁶¹ The combination therapy killed neuroblastoma cells with wild type ALK, amplified ALK, and even those that had activating ALK mutations. The sensitization of resistant models was also achieved. The most common side effects from Crizotinib treatment include visual effects (appearance of flashing or shimmering lights) and liver enzyme abnormalities.¹⁶² A more recent study on combination therapy blocked both ALK and MDM2, a ubiquitin ligase that targets the tumor suppressor protein p53 for degradation.¹⁵⁹ This study found that blocking ALK and MDM2 simultaneously could potentially overcome Crizotinib resistance as well. Admittedly, more research is required on these combination therapies to prove their effectiveness in neuroblastoma patients.

Promoting Differentiation

Retinoic acid promotes neuronal differentiation, but its lack of success in the treatment of high risk NB has driven researchers to find more effective combination therapies. A recent study by Westerlund et al observed the effects of 5-Aza-deoxycytidine (AZA) and retinoic acid on NB cell lines.¹⁶³ They found that AZA sensitizes the cells for retinoic acid treatment, consequently leading to decreased proliferation and increased differentiation. Tumors that were treated with AZA and retinoic acid were about 4 times smaller than those in the negative

control group, which were treated with DMSO. Another study by Rettig et al combined retinoic acid with HDAC8 inhibitors, which also resulted in increased differentiation and reduced levels of MYCN expression.¹⁶⁴ Experiments on mice revealed that exceeding the tolerable dose of 10 mg/kg per day led to severe abdominal swelling.

DISCUSSION

Neuroblastoma, like many other cancers, is a complex disease that involves various cellular mechanisms. MYCN amplification, ALK mutations, and structural and numerical alterations of the genome have been linked to the onset of this disease. Recent sequencing studies have identified even more genetic factors that drive NB including the occurrence of chromothripsis and changes in PTPN11, ATRX, ARID1A, ARID1B, and neuritogenesis genes. The discovery of these factors has helped researchers gain a better understanding of the disease pathway, but the exact mechanisms of action are still not entirely understood. Nonetheless, there have been improvements in the identification of high-risk patients and the development of therapies. Despite this progress in treatment, however, resistance to therapy, low efficacy, and toxicity have been some of the biggest barriers in the treatment of high-risk patients, who continue to exhibit survival rates that are lower than 50%.¹⁶⁵ Problems relating to drug resistance are being addressed by attempting several combination therapies that may

incorporate chemotherapy and immunotherapy drugs in order to target more than one component of the disease pathway. Some currently active clinical trials are testing an experimental drug Hu3F8 (an anti GD2-monoclonal antibody) in combination with other drugs including Irinotecan, Temozolomide, and GM-CSF.^{166,167,168} Other active immunotherapy trials are focusing on the combination of antibodies and natural killer T cells to target GD2.¹⁶⁹ Some researchers are also incorporating the use of retinoic acid and immunotherapy to simultaneously kill cancer cells and promote differentiation.¹⁷⁰ Evidently, immunotherapy testing has been a major focus for current trials because NB tumor cells, given their neuroectoderm origin, express unique biomarkers that can be used as potential targets. Thus, immunotherapy may prove to be less toxic than chemotherapy drugs.

The role of research and clinical trials is incredibly important in the treatment of patients. It was previously reported that approximately 76% of NB patients were enrolled in an active clinical trial.¹⁶⁵ Survival rates for neuroblastoma have consistently increased in the past decades and improvements may be seen in the coming years as current trials reach completion. Nonetheless, it is essential to continue exploring pathogenesis and regression mechanisms to further understand NB and keep improving treatment strategies and patient outcomes. Future advances in NB research could lead to the development of more detailed staging and risk analysis systems that will help healthcare professionals determine the best line of treatment for high-risk

patients and reduce treatment of low-risk patients who are likely to experience spontaneous regression.

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