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Chapter 10

Novel Therapeutic Approaches for Neuroblastoma

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http://dx.doi.org/10.5772/55451

1. Introduction

Neuroblastoma (NB) is the most common pediatric extracranial solid tumor of childhood, and 45% of patients have high-risk tumors, nearly all of which are metastatic (stage 4) when diagnosed [1]. Patients with neuroblastoma are risk stratified based on presenting factors including age, stage and location of disease, and specific biologic molecular markers of the tumor, including NMYC status and ploidy [1-3]. Treatment given is tailored to whether a patient has low, intermediate or high-risk disease. The overall prognosis for those with high risk or relapsed disease remains poor despite the standard therapies of surgery, radiation, and high dose chemotherapy followed by stem cell rescue. Additionally, many patients who survive suffer from complications related to their treatment. In this chapter, we review the literature that provides a rationale for the use of novel targeted agents to improve the treatment and survival while lessening toxicity of patients with neuroblastoma who have failed standard therapies.

In particular, we focus our discussion on a few specific signaling pathways. The central role of the phosphatidylinositol 3-kinase-Akt-phosphatase and tensin homolog (PI3K-Akt-PTEN) axis and RAF-MEK-ERK as potential molecular targets to control downstream effectors of coordinated cell division, tumor growth, angiogenesis, apoptosis, invasion and cellular metabolism in the tumor and surrounding stromal compartments. The PI3K and RAF-MEK-ERK pathways have also been implicated in modulating p53, the hypoxia-inducible factor 1 (HIF1α), mycN and others.

NMYC is known to play a role in the tumorigenesis of certain high-risk neuroblastoma tumors and its control has many implications in targeting therapy. Additional pathways and targets explored in this chapter are the RAS/Raf/MEK/ERK pathway, specific angiogenesis inhibitors including VEGF, ALK 1 mutations and inhibitors, and control of apoptosis through caspase 8.
We also discuss the idea of synthetic lethality and the concepts of sequential versus simultaneous inhibition. We will discuss the emerging importance of genomic and metabolomic profiling in tumor interrogation with therapeutic considerations.

We will review the literature supporting a role for cancer stem cells (CSCs) in the pathogenesis of neuroblastoma and the signaling pathways that define the CSC phenotype. We discuss the role targeted therapies in CSC related therapeutics and the adaptive responses that such cells have when exposed to targeted therapeutic agents.

Lastly, the emerging role of immunotherapeutics into both standard and targeted therapies for neuroblastoma is explored. This includes areas of T cell and macrophage infiltration of tumors, interleukin and cytokine involvement, and anti-GD2 human and mouse monoclonal antibodies.

2. PTEN and PI-3 kinase and mycN signaling as targets for NB therapeutics
   — The intercept node hypothesis

The idea that some signaling pathways are more central to tumorigenesis than others was suggested by our laboratory and others [4]. From connectivity map analysis, some signaling proteins appear connected to a large number of upstream and downstream effector pathways. These are considered central “intercept nodes” [4, 5] which provide coordinate control over the output of a large number of cell surface receptor input. The specificity of signaling downstream of such intercept nodes is generally fine tuned by more specialized signaling effector proteins e.g. Rac2, HIF1α, NFκB or mycN which encode more specific signaling content. Two such central pathways, PTEN-PI-3-AKT and Raf-MEK-ERK are critical for NB survival, proliferation, invasion and angiogenesis in vivo [6-9]. A large number of small and large pharmaceutical companies have developed small molecule inhibitors which block these two pathways. Considering the importance of mycN amplification in the pathogenesis of NB, and the role of PI-3K and MAP kinase in the GSK3β dependent regulation of mycN a number of investigators have determined the efficacy of PI-3 kinase inhibitors in NB models [9]. Despite evidence of efficacy no PI-3 kinase inhibitors have entered pediatric oncology clinical trials to date. One pan PI-3 kinase inhibitor, SF1126 is slated to enter pediatric oncology Phase I clinical trials in early 2013 [10]. Importantly, the tumor and stromal compartment share many of the same signaling pathways to regulate the process of tumorigenesis in vivo.

3. Role of angiogenesis in tumorigenicity of neuroblastoma / PI3 kinase and VEGF inhibitors in treatment of neuroblastoma

Work from a number of laboratories indicates that the angiogenic response is coordinately and highly regulated physiologic response to hypoxia and inflammation. Hence it is not surprising to learn that central node in mammalian cells control output from many cell surface receptors
to regulate this response [11]. We and others have shown that PTEN a major tumor suppressor protein regulates angiogenesis and loss of PTEN results in deregulation of PTEN and multiple downstream signaling pathways shown in Fig. 1 and 2 which have all been implicated in the literature to exert coordinate control of angiogenesis in vivo [4, 5, 12, 13].

In general, angiogenesis plays an important role in the progression and metastasis of malignant tumors [14]. In neuroblastoma, tumor vascularity is correlated with an aggressive phenotype [15, 16]. Pro-angiogenic factors are differentially expressed in high-risk neuroblastoma [17, 18]. Vascular endothelial growth factor (VEGF) is a specific endothelial cell mitogen that stimulates angiogenesis and plays a crucial role in tumor growth [19]. Over expression of VEGF has been demonstrated in neuroblastoma, nephroblastoma, as well as in various other cancers [20-22]. Recent studies have validated inhibition of VEGF as an effective antiangiogenic therapy in some of these cancers [23-25]. Although several preliminary studies have demonstrated that expression of angiogenic growth factors, including VEGF, correlate with a high-risk phenotype in neuroblastoma, clinical data are still insufficient to draw conclusions [17, 21, 26, 27]. Therefore, further clinical studies, are needed to evaluate the possible significance of these factors for use in a routine clinical practice. Preclinical studies also suggest that antiangiogenic strategies may be effective in the treatment of neuroblastoma [28]. In addition, phase I clinical trials (COG study) using the human anti-VEGF antibody, bevacizumab, in pediatric patients with refractory solid tumors reported promising results [29]. Recently, Jakovljevic et al. has determined VEGF expression by immunohistochemistry using anti-VEGF antibody in

Figure 1. The PI3K–Akt–PTEN intercept node. As shown, a large number of growth factor receptors (GFR) of which TrkB is an oncogene in NB would feed into the central node to activate PI-3 kinase, AKT and/or Raf-MEK-ERK pathways. Downstream subnodes encode specificity e.g. GSK3b, MDM2, mycN, Rac2, etc. Major tumor suppressors like PTEN and p53 control output from these two central nodes. MDM2 regulates p53 in an AKT dependent manner; RAF-MEK-ERK and AKT regulate GSK3b to control mycN stability and transcriptional activity.
paraffin embedded primary tumor tissue from 56 neuroblastoma patients and reported that VEGF expression correlated with disease stage and survival in neuroblastoma patients [30]. Whether inhibition of angiogenesis is a realistic approach for preventing dissemination of neuroblastoma remains to be determined, but we can suggest that inhibitors of VEGF can be used in the treatment of neuroblastoma. Finally, we suggest that the more global inhibition of PI3 kinase or combined PI3K/MEK inhibition would provide a more potent antiangiogenic modality to block tumor induced angiogenesis in this disease.

4. Cancer stem cells in neuroblastoma tumorigenicity

The Cancer Stem Cell Theory postulates that tumors contain a subset of cells that are capable of increased self-renewal and differentiation, can propagate tumor growth and are resistant to apoptosis [31, 32]. These stem-like cancer cells are analogous to normal stem cells [33] but differentiate into diverse cancer cells that form the major portion of the tumor. Recent evidence suggests the presence of stem cells in various cancers including those of the blood [34], breast [35], prostate [36] and brain [37].

Evidence for the presence of cancer stem cells in brain tumors first came from the observation that human medulloblastoma, astrocytomas, and ependymomas contain cells that express the neural stem cell marker CD133 [38] [39]. Singh et al. [37] have shown that human brain tumors contain CD133+ stem-like cells that are capable of growing tumors in immune-deficient mice. Cournoyer et al.[40] have shown that CD133 high neuroblastoma (NB) cells have high tumor initiating cell properties, and Coulon et al.[41] suggest that CD133, ABC transporter, Wnt and NOTCH genes are sphere markers in NB cells. Overall, 19–29 % of cells in glioblastomas and 6–21 % of cells of medulloblastomas are reported to be CD133+ and tumorigenic [33]. Recently, several groups have suggested that CD15 (stage specific embryonic antigen 1 or SSEA-1), which is expressed on neural progenitor and stem cells, may be a better marker than CD133 of tumor-initiating cells in MB, glioma, and ependymoma [42-44]. Hansford et al has recently identified tumor initiating cells from NB bone marrow metastases that have several properties of cancer stem cells including the expression of stem cell markers, the ability to self renew and the capability to form metastatic NB in immunodeficient animals with as few as 10 cells [45]. Kaplan’s laboratory has further defined the NB tumor initiating cell (TIC) with stem cell like properties to express, CD133 and CD44. These cells isolated from NB bone marrow have tumor initiating activity and upon profiling display sensitivity to a number of targeted therapeutic agents.

A key aspect of the tumor stem cell (TSC) niche is the balance of signals received, and over recent years considerable attention has been directed towards understanding the role of signaling pathways, which are critical mediators of normal stem cell biology, in cancers. The embryonic signaling pathways most commonly implicated in tumorigenesis include Hedgehog, Notch, and Wnt pathways. Sonic Hedgehog (SHH) signaling is important in embryonic cell development and proliferation and aberrant pathway activation can lead to tumor formation, tumor cell self-renewal and the development of metastatic disease [48]. Similarly,
Notch plays a crucial role in biological functions of development and cell fate including cell differentiation and proliferation [49]. Constitutive activation of Notch can lead to tumorigenesis and cell survival, and Notch activity is involved in tumor angiogenesis [50].

In tumor compartment, PI3K–Akt–PTEN intercept node is a central regulator of survival, proliferation, invasion and angiogenesis in Neuroblastoma. PI3K controls PIP3 levels, thereby regulating lipid-associated second messenger output from upstream effectors. PI3K and Akt can be activated by many cell surface receptors. Akt becomes locked in an active conformation and phosphorylates numerous proteins involved in growth and survival, cellular metabolism, stress response and angiogenesis. Akt modulates phosphorylation of GSK3β and relieves tonic inhibition of c-Myc and cyclin D to promote cell survival [46]. Akt contributes to the Warburg effect by inducing HIF1α transcription and stimulating aerobic glycolysis. Intratumoral hypoxia also drives angiogenesis through transcription of proangiogenic genes including VEGF and PDGF. Tumor angiogenesis is promoted by Akt-mediated phosphorylation of MDM2. Activated MDM2 translocates from the cytoplasm to the nucleus, where it binds p53, targeting it for ubiquitination and degradation. This process prevents p53 from exerting its antiangiogenic effect. A more effective strategy might be to modulate tumor growth and angiogenesis by targeting major signaling nodes such as the p53–MDM2 or PI3K–Akt–PTEN nodes with agents such as Nutlin 3A or with PI3K inhibitors (e.g. PI-103, BEZ-235 or SF1126), respectively. Abbreviations: GSK3β, glycogen synthase kinase 3 β; HIF1α, hypoxia inducible factor 1α; MDM2, mammalian double minute 2; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PI-103, BEZ-235 or SF1126.

In stromal compartment, the major cellular pathways of the immune response which may have anti- or pro-tumor effects are shown. NK cells and CD8+ CTLs may directly target tumor cells for lysis; however this may be countered by decreased tumor expression of NKG2D ligands or MHC class I. Dendritic cells are important for priming an anti-tumor immune response, although immature DCs and IDO-expressing DCs may instead lead to the induction of tolerance. Myeloid-derived suppressor cells and regulatory T cells (Treg) may also suppress the anti-tumor CTL response. TH1 cells and M1 macrophages produce proinflammatory cytokines which help to stimulate the anti-tumor immune response, whilst TH2 cells (and other cell types) produce IL-10 which may have a predominantly inhibitory effect on the anti-tumor response. Tumor-associated M2 macrophages may promote tumor growth and metastases via a number of different mechanisms. Figure adopted from Morgenstern et al [47].

Figure 2. Signaling and cellular pathways controlling tumorigenicity of Neuroblastoma. In tumor compartment, PI3K–Akt–PTEN intercept node is a central regulator of survival, proliferation, invasion and angiogenesis in Neuroblastoma. PI3K controls PIP3 levels, thereby regulating lipid-associated second messenger output from upstream effectors. PI3K and Akt can be activated by many cell surface receptors. Akt becomes locked in an active conformation and phosphorylates numerous proteins involved in growth and survival, cellular metabolism, stress response and angiogenesis. Akt modulates phosphorylation of GSK3β and relieves tonic inhibition of c-Myc and cyclin D to promote cell survival [46]. Akt contributes to the Warburg effect by inducing HIF1α transcription and stimulating aerobic glycolysis. Intratumoral hypoxia also drives angiogenesis through transcription of proangiogenic genes including VEGF and PDGF. Tumor angiogenesis is promoted by Akt-mediated phosphorylation of MDM2. Activated MDM2 translocates from the cytoplasm to the nucleus, where it binds p53, targeting it for ubiquitination and degradation. This process prevents p53 from exerting its antiangiogenic effect. A more effective strategy might be to modulate tumor growth and angiogenesis by targeting major signaling nodes such as the p53–MDM2 or PI3K–Akt–PTEN nodes with agents such as Nutlin 3A or with PI3K inhibitors (e.g. PI-103, BEZ-235 or SF1126), respectively. Abbreviations: GSK3β, glycogen synthase kinase 3 β; HIF1α, hypoxia inducible factor 1α; MDM2, mammalian double minute 2; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase; PI-103, BEZ-235 or SF1126.

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family proteins help direct a wide range of developmental processes including cell fate, proliferation, motility, and polarity [51]. Dysregulation of the Wnt pathways has been implicated in tumor formation, proliferation, and maintenance [52]. All of the current pediatric studies demonstrating that progenitor and stem cells can respond to embryonic signaling have been in MB or primitive neuroectodermal tumors (PNET). Aberrant SHH signaling has been implicated in MB, and recently was used to define one of four distinct molecular variants of MB [53].

In order to identify pathways required for proliferation and cell survival characteristics of TIC in neuroblastoma, Grinshtein et al. has performed drug screen on bone marrow derived tumor initiating cells (TICs) with a unique collection of pharmacological inhibitors. They identified that PI3K (phosphoinositide 3 kinase)/AKT, PKC (protein kinase C), Aurora, ErbB2, Trk and Polo-like kinase 1 (PLK1) are the potential kinase targets for survival of TIC [54]. Their studies demonstrated that PLK1 inhibitors are an attractive candidate therapy for metastatic NB. Another group suggested that both PI-3 kinase as well as Ras-RAF-MEK-Erk signaling pathways promote the tumorigenicity of the glioblastoma cancer stem like cells, and combined treatment with MEK and PI-3 kinase inhibitors can block the differentiation of glioblastoma cancer stem like cell into non tumor initiating status [55].

The therapeutic resistance of cancer stem cell to current treatment modalities such as chemotherapy and radiation make these cells clinically relevant irrespective of their origin. Resistance to chemotherapeutic agents has been demonstrated in neuroblastoma stem cells and sarcoma stem cells including Ewing’s sarcoma and osteosarcoma. Recent work by Hambardzumyan suggests that the PI-3 kinase pathway activity promotes post-radiation survival in cancer stem cells in medulloblastoma [67]. Although lots of literatures are available on the cancer stem cell in neuroblastoma but yet the novel signaling pathways controlling the proliferation and survival of cancer stem cell and the mechanism behind resistance developed due to chemotherapy needs to be investigated.

5. Neuroblastoma and cancer metabolism

It has been known from a long time that cancer cells take up and metabolize glucose and glutamine to a degree that far exceeds their needs for these molecules in anabolic macromolecular synthesis [56]. Commonly occurring oncogenic signal transduction pathways initiated by receptor tyrosine kinases or Ras engage PI3K-Akt signaling to directly stimulate glycolytic metabolism under aerobic conditions a condition termed the Warburg and Pasteur effects [56-58]. Myc-activation/amplification is one of the most common oncogenic events observed in cancer and is known to drive the progression of a certain subgroup of neuroblastoma [59]. The activation of mycN could occur through amplification of the mycN gene or through upstream activation of signaling pathways that would stabilize mycN e.g. trkB, IGF-1 or the activation of Raf and/or PI-3K-AKT stimulation. Oncogenic levels of Myc have recently been linked to increased glutaminolysis through a coordinated transcriptional program program [60-62]. Quantitative RTPCR and ChIP experiments support Myc’s binding and transcriptional
activation of two high affinity glutamine transporters: SLC38A5 (also called SN2) and SLC1A5 (ASCT2), the transporter required for glutamine-dependent mTORC1 activation [60, 63]. In addition to facilitating glutamine uptake, Myc promotes the metabolism of imported glutamine into glutamic acid and ultimately into lactic acid [60]. Whether the tendency of Myc to complement Ras and PI3K-Akt [64, 65] is related to the interdependence of glutamine and glucose metabolism in support of cell growth remains an open question. The work of C. Dang and other points to a potential important metabolic requirement for glutamine in c-myc and mycN driven tumors where glutamine can serve a role in promoting tumor growth [58, 66]. This might suggest a role of agents which deplete glutamine (glutaminases) as a therapeutic target for mycN driven malignancies like neuroblastoma and the SHH subtype of medulloblastoma.

6. Role of tumor infiltrating immune cells in tumorigenicity of neuroblastoma

Solid tumors are composed of tumor stromal cells, blood vessels, infiltrating immune cells and tumor cells themselves. Over the last decade, a growing body of literature has highlighted the importance of the tumor microenvironment for the prognosis of different types of cancer [68]. The tumor microenvironment contains many resident cell types, such as adipocytes and fibroblasts, but it is also populated by migratory hematopoietic cells, including lymphoid cells, granulocytes, mast cells, dendritic cells, natural killer cells, neutrophils and macrophages. These haematopoietic cells have pivotal roles in the progression and metastasis of tumors [69, 70]. The significance of tumor stroma for the overall prognosis may be in part due to the fact that several components of the tumor-microenvironment have been shown to compromise immune effect functions against tumor cells [71]. The concept of tumor-promoting inflammation is a recognized enabling characteristic of cancers [72].

The first evidence suggesting immune responses to neuroblastoma was provided in 1968 when blood leukocytes, which were 50–70% lymphocytes, were reported to inhibit colony formation by neuroblastoma cells [73]. These lymphocytes inhibited colony formation by both autologous and allogeneic neuroblastoma cells but did not affect growth of fibroblasts from the same donors. Plasma from these patients also was reported to inhibit tumor cell colony formation in the presence of complement. In this same time, primary tumors were reported to contain leukocytes [74, 75], and some localized and metastatic neuroblastomas were reported to regress spontaneously [76, 77]. Together, these studies suggest that the immune system could develop an anti-neuroblastoma response. In this section, we will highlight the role of tumor infiltrating immune cells in progression of this disease and how blocking the function of these infiltrating cells may prove beneficial in its treatment of NB.

a. Tumor infiltrating Lymphocytes

Tumor-associated lymphocyte population includes CD8+ cytotoxic T cells, CD4+ T helper cells, regulatory T cells (Tregs), NKT or γδT cells. Tregs are immunosuppressive regulatory T cells. Tregs are able to suppress the activity of CTLs by direct cell-cell contact and also secrete
immunoregulatory cytokines such as transforming growth factor β (TGF-β) and interleukin-10 (IL-10). However, the role of Tregs is much less clear and to our knowledge there are no published data on the presence (or otherwise) of Tregs in pediatric tumors.

CD8+ cytotoxic T lymphocytes (CTL) are a primary source of anti-tumor activity in the immune system [1, 3]. In many adult cancers the presence of significant numbers of tumor-infiltrating lymphocytes, potentially represents the host immune response against the tumor and is associated with improved prognosis [78-80]. In neuroblastoma, Martin *et al.* [81] suggested a correlation between lymphocyte infiltration and improved survival, although these data are confounded by tumor grade since lymphocytic infiltrates were seen more frequently in low grade, differentiating tumors. In a separate examination of 26 high-risk neuroblastoma tumor samples, there was minimal or undetectable infiltration of CD8+ or CD4+ T cells, CD20+ B cells or CD56+ NK cells within tumor nests [82], although in most patients CD8+ or CD4+ lymphocytes were present within the peritumoral stroma. Interestingly, the majority of patients had evidence of small numbers of circulating cytotoxic T cells against the tumor antigen survivin (expressed by all of the tumors in this study) and these CTLs were highly functional in *in vitro* assays [82]. The experiments conducted by another group in NXS2 murine neuroblastoma model have shown that oral vaccination with a survivin DNA minigene was associated with increased target cell lysis, increased presence of CD8(+) T-cells at the primary tumor site, and enhanced production of pro-inflammatory cytokines [83]. Another pre-clinical study have demonstrated that tyrosine hydroxylase and MYCN proteins, which are relatively specific for neuroblastoma cells compared to normal cells, include peptides that can be targets for CTL. Vaccination of mice with tyrosine hydroxylase DNA minigenes can induce CTLs, eradicate established primary NXS2 neuroblastoma tumors, and inhibit spontaneous metastases without induction of autoimmunity [84, 85].

However, despite these cellular responses to NB, the presence of tumor-infiltrating CTL is rare, suggesting a block in T cell trafficking that may protect the tumor from CTL-mediated cytotoxicity. Therefore, strategies aiming to generate CTLs must take into account mechanisms by which neuroblastoma cells may avoid immune elimination. These include decreased expression of peptide presenting HLA class I molecules by tumor cells, which can impair target peptide recognition by CTLs [82, 86, 87]. Also, neuroblastoma cells express low levels of antigen processing genes, including LMP-2, LMP-7, and TAP-1, which are necessary for preparation of peptides from proteins for presentation by HLA class I molecules to CTLs [88, 89]. Neuroblastoma cells also induce monocytes to release HLA-G, which suppresses both CTL and NK mediated cytotoxicity by interacting with inhibitory receptors or inducing apoptosis via CD8 ligation or the Fas-FasL pathway [90]. Thus, effective CTL anti-tumor responses require that these escape mechanisms be evaluated and, if present, be overcome.

**b. Natural Killer Cells**

Natural Killer (NK) cells represent a particular subset of T lymphocytes, which express both T cell markers, such as the αβ T-cell receptor (TCR) and associated CD3 complex, and NK cell markers, such as NK1.1[91]. These cells recognize glycolipids presented by the MHC class I-like molecule CD1d and are believed to play an important role at the interface between the
innate and adaptive immune responses to infection and malignancy [92]. Two main subtypes of NKT cell are recognised, with Type I NKT cells expressing an invariant α-TCR chain and being implicated in antitumor immunity, whilst Type II NKT cells express a variety of TCR molecules (in addition to CD1d) and appear to have a more immune inhibitory role [91]. The presence of these immune effector cells within tumors has been examined in a number of different malignancies, including, neuroblastoma. Type I NKT cells were found in 53% of 98 untreated primary stage 4 neuroblastoma samples [93] and their infiltration correlated with favorable outcome, with expression of the chemokine CCL2 and with absence of MYCN amplification (indicating less aggressive disease). Subsequent investigations have confirmed that expression of CCL2 is repressed in MYCN amplified tumors, leading to a failure of NKT cell infiltration and potentially contributing to tumor immune escape [94].

Recent studies have suggested anti-tumor role of NK cells in high risk neuroblastoma NK cells are activated to be cytotoxic and secrete IFNγ by IL-2. IL-2 alone has been tested in phase I and II trials for patients with neuroblastoma, and, although immune effects were documented, no objective tumor responses were observed [95, 96]. Lenalidomide is an immune modulating drug that activates T cells to secrete IL-2, which in turn activates NK cell cytotoxicity and ADCC [97, 98]. Clinical trials in children and adults demonstrated increased numbers of NK cells and cytotoxicity, decreased T regulatory cells, and increased secretion of IL-2, IL-15, and GM-CSF after 21 days of lenalidomide treatment [99, 100]. Thus, lenalidomide may be useful for activating NK cells to enhance mAb immunotherapy of neuroblastoma.

c. Role of tumor associated macrophages

Macrophages represent a further important cellular component of the tumor stroma. Far from being mere bystanders to tumor development, there is increasing evidence that tumor-associated macrophages (TAMs) promote and facilitate tumor growth [101, 102]. Of key importance is the concept of distinct macrophage phenotypes, mirroring the dichotomy between Th1 and Th2 T helper cells and type I and type II immune responses. Alternatively activated M2-macrophages are involved in polarized Th2 inflammatory reactions and characterized by expression of arginase-1 and mannose and scavenger receptors [103, 104]. On the other extreme, classically activated M1 macrophages are IL-12 high, IL-23 high, IL-10 low; produce high levels of inducible nitric oxide synthetase (iNOS); secrete inflammatory cytokines such as IL-1β, IL-6, and TNF; and are inducer and effector cells in Th1 type inflammatory responses [105]. It has been suggested that tumor-associated macrophages (TAMs) display an M2-like phenotype [106].

TAMs are recruited to tumors when stimulated by growth factors and chemokines, produced by the tumor cells [107, 108]. The conventional wisdom about TAM function is that they are recruited to reject the tumor, which has been recognized as foreign because tumors express unique antigens. However, there is a growing body of evidence that the tumor microenvironment is immunosuppressive [109], perhaps as a result of selection for such an environment a process recently termed ‘immunoediting. Recent data indicate that TGF-β1 has an important role in suppressing these local responses and that inhibiting this molecule can result in tumor rejection [110, 111]. It is noteworthy that TAMs can both produce TGF-β1 and process latent
TGF-βs to produce their active forms[111]. In addition, the local cytokine milieu in the tumor tends to block the immunological functions of these newly recruited mononuclear phagocytes such as antigen presentation and cytotoxicity towards tumors, and diverts them towards specialized TAMs that are immunosuppressed and trophic [112]. A principal component of this cytokine mixture is CSF-1, which locally blocks the maturation of dendritic cells so that they are unable to present antigens and promotes the development of immunosuppressed trophic TAMs. TAMs promote tumor growth by affecting angiogenesis, immune suppression, invasion and metastasis [101, 102]. Existing literature suggests that tumor associated macrophages secrete several genes including matrix metalloproteinases-9 (MMP-9) [113], urokinase-type plasminogen activator (uPA) [114], vascular endothelial growth factor (VEGF) [115], and cyclooxygenase-2 (Cox-2) [116] which promotes tumor growth by breaking down extracellular matrix. The role of TAMs in tumor growth and progression is highlighted in Figure 3.

**Figure 3. The role of TAMs in tumor growth, invasion and metastasis:** Tumor-derived chemokines, cytokines and vascular endothelial growth factor (VEGF), actively recruit circulating blood monocytes at the tumor site in the tumor micro-environment monocytes differentiate into tumor-associated macrophages (TAM), where they promote tumor growth and metastasis and establish a symbiotic relationship with tumor cells. The above tumor-derived factors positively modulate TAM survival. TAMs also secrete growth factors, which promote tumor cell proliferation and survival, regulate matrix deposition and remodeling and activate neo-angiogenesis. Figure modified and adapted from Sica et al. [106]

Clinical studies have, on balance, shown a correlation between an abundance of TAMs and poor prognosis [108]. These data are particularly strong for breast, prostate, pancreatic, ovarian and cervical cancers; the data for stomach and lung cancers are contradictory [108, 117, 118], and in a small study in colorectal cancer, their presence was associated with good prognosis [119]. However, taking all reports into account regardless of method and sample number more than 80% show a significant correlation between TAM density and poor prognosis, whereas less than 10% associate TAM density with a good prognosis [108]. So, increased TAM density
is usually associated with advanced tumor progression and metastasis in most of the cancers. However, the prognostic significance of tumor-associated inflammatory cells in metastatic disease and in childhood cancers is mostly unknown.

Recent reports suggest that interaction between tumor and inflammatory cells contribute to the clinical metastatic neuroblastoma phenotype [120]. It has been reported that metastatic neuroblastomas had higher infiltration of TAMs than loco regional tumors, and metastatic tumors diagnosed in patients at age ≥ 18 months had higher expression of inflammation related genes than those in patients diagnosed at age < 18 months. They identified 14 genes, out of which nine were tumor cell related and five were inflammation related that comprises a prognostic signature for neuroblastoma. Expression of inflammation related genes representing TAMs (CD33/CD16/IL6R/IL10/FCGR3) contributed to 25% of the accuracy of a novel 14-gene tumor classification score [120]. Another study by Song et al., demonstrated that CD1d+ TAMs promote neuroblastoma growth via IL-6 production and that expression of monocyte/macrophage markers, CD14/CD16, and IL-6 or IL-6R inversely correlates with long-term disease-free survival in patients with stage 4 MYCN–non-amplified neuroblastoma [121]. They suggested that cotransfer of human monocytes and NKTs to tumor-bearing NOD/SCID mice decreased monocyte number at the tumor site and suppressed tumor growth compared with mice transferred with monocytes alone. Thus killing of TAMs can be suggested as a novel mechanism of NKT antitumor activity that relates to the disease outcome. Although less is known about the role of stromal compartment in tumorigenicity in neuroblastoma and other childhood tumors but recent reports suggesting infiltration of macrophages in metastatic neuroblastoma opened new opportunities to target tumor associated immune system cells in childhood cancer. It is unclear whether these TAMs represent M2 macrophages and the mechanisms that control macrophage differentiation along the M1 vs the M2 macrophage in tumor biology.

7. Multiple ‘Omics’ analysis an emerging concept in treatment of neuroblastoma

The “Omics” is a neologism widely adopted by scientists to refer to large scale analysis of genes (genomic), proteins (proteomics) and lately small metabolites (metabolomics). Modern molecular achievements over the last decade have seen the increase and implementation of multiple ‘omics’ technologies in oncology that promises to provide for a deeper comprehension of complex tumor pathways. It is believed that an integration of multiple “omics” technologies is likely to provide even further insight into the holistic view of the biology networks [122]. The studies of global expression profiles of both mRNA and protein are necessary to reveal the important pathways for an enigmatic disease such as neuroblastoma. During the past several years many studies utilized microarray-based high throughput technologies to investigate gene expression profiles and DNA copy number alterations in neuroblastoma [123, 124]. Guo et al. has performed exon array profiling to investigate global alternative splicing pattern of 47 neuroblastoma samples in stage 1 and stage 4 with normal or amplified MYCN copy number (stage 1-, 4- and 4+) Their results demonstrated a significant role of alternative splicing in high stage neuroblastoma and suggested a MYCN-associated splicing regulation pathway in stage 4+
tumors [125]. Studies from other groups have measured copy number alterations in a representative set of 82 diagnostic tumors on a customized high-resolution BAC array based CGH platform supplemented with additional clones across 1p36, 2p24, 3p21-22, 11q14-24, and 16p12-13, and integrated these data with RNA expression data [126]. They used an unbiased statistical method to define a set of minimal common regions (MCRs) of aberration and on the basis of unsupervised hierarchal clustering they identified four distinct genomic subclasses. These genomic subsets were highly correlated with patient outcome, and individual MCRs remained prognostic in a multivariable model. These studies mentioned above identified prognostic markers and genomic alterations specific to high-risk neuroblastoma, and showed the capability of identifying signatures which predict patient outcome. Since mRNA expression is not always indicative of corresponding protein expression because the abundance of specific proteins can be controlled by post-transcriptional translation and post-translational modifications, therefore the use of proteomics will help in detecting directly the actual biological effector molecules and should provide more accurate functional information about biological systems. With this idea, Chen et al. has performed parallel global protein and mRNA expression profiling on NB tumors of stage4 MYCN-amplified (4+) and stage1 MYCN-not-amplified (1-) using isotope-coded affinity tags (ICAT) and Affymetrix U133plus2 microarray respectively [127]. Pathway analysis of the differentially expressed proteins conducted by this group showed the enrichment of glycolysis, DNA replication and cell cycle processes in the upregulated proteins and cell adhesion, nervous system development and cell differentiation processes in the down-regulated proteins in 4+ tumors; suggesting a less mature neural and a more invasive phenotype of 4+ tumor.

Metabolomics falls behind its predecessor genomics and proteomics, but represent a burgeoning field with potential to fill up the gap between genotype and phenotype [128]. The high throughput nature of metabolomics makes it an attractive tool for scientists involved in the process of drug development. The reason for that lies in the principle that a patient's response to drugs and toxicities do not depend only on a person genetic make-up, but it is rather a factorial outcome of interactions between intrinsic factors and environment [128]. Therefore, metabolomics technology is a powerful tool that can accurately measure the entire spectrum of biochemical changes and mapping these changes to metabolic pathways [128, 129]. In 1995, Florian et al. [130] determined the metabolic characteristics of three types of human brain and nervous system tumors by high-resolution in vitro MRS and chromatographic analysis. Signals from leucine, isoleucine, glycine, valine, threonine, lactate, acetate, glutamate, and choline-containing compounds were similarly detected in meningiomas, glioblastomas, and NB. In 2007, Peet et al. [131] reported the results of in vitro 1H high-resolution magic angle spinning NMR spectroscopy (HRMAS) investigations performed on cell suspension of 13 lines of NB possessing multiple genetic alterations. In their study, a specific metabolite profile associated with MYCN-amplified and non-amplified tumor subtypes was described. Phosphocholine and taurine concentration ratios relative to total choline were found to be significantly more elevated in the MYCN-amplified as compared to the MYCN-non-amplified cell lines, and suggested that choline and taurine molecular pathways could be potential therapeutic targets in NB [131]. Recently, Imperiale et al. has characterized the metabolic content of intact biopsy samples obtained from 12 patients suffering from neuroblastoma by using (HRMAS) [132].
Their studies suggested that NB patients younger than 12 months contained a higher level of acetate and lysine. Conversely, higher amounts of glutathione, glutamate, myoinositol glycine, serine and ascorbic acid were detected in NB samples belonging to younger children.

Overall, the emerging concept of analyzing NB-specific ‘omics profiles to better understand and define the behavior of advanced-stage tumors along with providing direct and targeted therapy may ultimately translate into improved outcomes for high-risk NB.

8. Antibody dependent cellular toxicity (ADCC) / Role of ITAM and ITIM signaling in neuroblastoma

The Fcγ receptors (FcγRs) expressed on hematopoietic cells play a key role in immune defenses by linking humoral and cellular immunity [133]. FcγRs display coordinate and opposing roles in immune responses depending on their cytoplasmic region and/or their associated chains. Indeed, the activating receptors contain an immunoreceptor tyrosine-based activation motif (ITAM) and initiate inflammatory, cytolytic, and phagocytic activities of immune effector cells. In contrast, the inhibitory receptors that downmodulate the immune responses contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) [134, 135]. There are numerous Fc receptors for IgG (FcγR) that are widely expressed on immune cells. The FcγR family consists of four classes of receptors, FcγRI, FcγRII, FcγRIII, and FcγRIV, that have been identified in both mice and humans. There are significant similarities in the functions of the FcγR receptors between mice and humans, but there is limited homology in receptors themselves [136]. To date, only one inhibitory FcγR, FcγRIIb, has been identified and is the only receptor to have complete homology between mice and humans [136]. FcγRs can be found on virtually all hematopoietic cells except T cells; in most cases, cells coexpress activating and inhibitory FcγR, allowing for the balance between activating and inhibitory receptors to dictate their response [136]. NK cells are an exception to this rule and express only the activating FcγRIIIa. NK cells do not express the inhibitory FcγRIIb.

Antibodies directed against neoplastic cells provide new therapeutic approaches against various malignancies, including lymphoma, leukemia, melanoma, and breast and colorectal carcinoma [137, 138]. There is increasing evidence that the Fc portion of the anti-tumor IgG is a major component of their therapeutic activity, along with other mechanisms such as activation of apoptosis, blockade of signaling pathways, or masking of tumor antigens. Thus, by binding to activating FcγRs expressed by immune effector cells, such as macrophages, monocytes, neutrophils, or NK cells, tumor-specific antibodies trigger the destruction of malignant cells via antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis [139, 140].

Because of their rapid and unopposed responses to mAb, NK cells play a major role in the anti-tumor response elicited by tumor-specific mAbs. Multiple clinically successful mAbs utilize NK-mediated ADCC as a mechanism of action. Rituximab (anti-CD20), Herceptin (anti-Her-2/neu), Cetuximab (anti-EGFR), and the anti-GD2-mAbs 3F8 and ch14.18 are examples of tumor-specific mAbs whose clinical activity can be attributed, at least in part, to NK cells. Natural killer (NK) cells are powerful effector cells that can be directed to eliminate tumor cells through
tumor-targeted monoclonal antibodies (mAbs). Some tumor-targeted mAbs have been successfully applied in the clinic and are included in the standard of care for certain malignancies. Strategies to augment the antitumor response by NK cells have led to an increased understanding of how to improve their effector responses. Next-generation reagents, such as molecularly modified mAbs and mAb-cytokine fusion proteins (immunocytokines, ICs) designed to augment NK-mediated killing, are showing promise in preclinical and some clinical settings. Continued research into the antitumor effects induced by NK cells and tumor-targeted mAbs suggests that additional intrinsic and extrinsic factors may influence the antitumor response. Therefore more research is needed that focuses on evaluating which NK cell and tumor criteria are best predictive of a clinical response and which combination immunotherapy regimens to pursue for distinct clinical settings.

9. Tumor associated gangliosides / GD2 monoclonal antibodies

Gangliosides (GD) are membrane-associated glycosphingolipids which have important regulatory roles during embryogenesis and have also been implicated in tumor development. Particular gangliosides, which show restricted patterns of expression in normal tissue, may be expressed at high levels by tumor cells (e.g. GD3 by melanoma) and are implicated both in tumorigenesis and as mediators of metastatic spread [141]. There is also evidence that gangliosides secreted by tumor cells can modulate the immune response and, in particular, act to inhibit dendritic cell differentiation and function. Neuroblastoma (and other neuroendocrine) tumor cells ubiquitously express the ganglioside GD2, whilst expression in normal tissues is restricted to neurons. Thus, GD2 is an attractive antigen for neuroblastoma immunotherapy strategies [142] including humanized anti-GD2 monoclonal antibodies such as ch14.18 [143], or GD2-directed cytotoxic lymphocytes. A chimeric human–murine anti-GD2 monoclonal antibody [144] called ch14.18 has shown activity against neuroblastoma in preclinical studies [145] and early-phase clinical trials [146, 147], this activity could be enhanced when ch14.18 is used in combination with granulocyte–macrophage colony-stimulating factor (GM-CSF) [148] or interleukin-2 [149, 150] to augment antibody-dependent cell-mediated cytotoxicity. The feasibility of administering ch14.18 in combination with GM-CSF, interleukin-2, and isotretinoin during the early post-transplantation period has been shown in two sequential pilot phase 1 studies [143, 151]. This progression of clinical trials culminated in the recently completed phase III randomized study of isotretinoin together with ch14.18, IL-2, and GMCSF vs. isotretinoin only for children with high-risk neuroblastoma who had a clinical response to induction therapy and myeloablative consolidation therapy/AHSCT. Immuno-therapy after consolidation significantly improve event free survival (EFS) (66 ±5% vs. 46 ±5% at 2 years, P = 0.01) and overall survival (86 ± 4% vs. 75 ± 5% at 2 years, P = 0.02). This was the first demonstration that antibody based therapy improves EFS and overall survival. Although EFS was improved by adding immunotherapy to isotretinoin, approximately 40% of patients still relapsed during or after this therapy [152]. Additionally, the combination of ch14.18 with IL-2 and GM-CSF has significant toxicities, including neuropathic pain, fever without neutro-
penia, infection, hypokalemia, hypotension, and capillary leak syndrome. Thus, a search for new agents to combine with ch14.18 to improve efficacy and decrease toxicity is justified.

Immunocytokines commonly known as antibody-cytokine fusion proteins combine the targeting ability of antibodies with the functional activity of cytokines, and are known to improve antibody-based therapy by delivering cytokines to the microenvironment to both activate effector cells and modulate the microenvironment. To date, immunocytokine research has focused on ADCC mediated by NK cells and on induction of CTL. An anti-GD2/IL-2 immunocytokine eradicated hepatic metastases of neuroblastomas in SCID mice that had been reconstituted with human lymphokine (IL-2) activated killer cells [153, 154]. In contrast, the combination of monoclonal anti-GD2 antibody and IL-2 at doses equivalent to the immunocytokine only reduced tumor load. In a syngeneic murine model of GD2 expressing melanoma, targeting with an anti-GD2 antibody/IL-2 immunocytokine resulted in generation of CD8+ T lymphocytes that could eradicate tumor as well as prevent tumor growth [154]. Based upon these data, phase I and II studies have tested a humanized anti-GD2/IL-2 immunocytokine (hu14.18/IL-2) in patients with refractory or relapsed neuroblastoma. In the phase I study of 27 patients, treatment with hu14.18/IL2 caused elevated serum levels of soluble IL-2 receptor alpha (sIL2Rα) and lymphocytosis. There were no measurable complete or partial responses to hu14.18/IL2; however, three patients showed evidence of antitumor activity [155]. In the phase II study, 39 patients with recurrent or refractory neuroblastoma were enrolled (36 evaluable). No responses were seen for patients with disease measurable by standard radiographic criteria (stratum 1) (n = 13). Of 23 patients with disease evaluable only by 123I-metaiodobenzylguanidine (MIBG) scintigraphy and/or bone marrow histology (stratum 2), five patients (21.7%) responded; all had a complete response of 9, 13, 20, 30, and 35+ months duration. Grade 3 and 4 non-hematologic toxicities included capillary leak, hypoxia, pain, rash, allergic reaction, elevated transaminases, and hyperbilirubinemia, which were reversible within a few days of completing a treatment course. These results support further testing of hu14.18/IL2 in children with non-bulky high-risk neuroblastoma [156].

10. Summary

Herein, we have reviewed a number of important areas of basic and translational research related to emerging novel therapies for the pediatric solid tumor, neuroblastoma. These include: 1) Signaling pathways within the tumor cell itself e.g. oncogenes and tumor suppressor proteins 2) Signaling pathways that regulate the tumor stromal compartment to control angiogenesis and the immune system and 3) Elements of cancer metabolism related to the oncogene addiction hypothesis.

Future studies will tap into these areas of basic science investigation to illuminate new avenues for therapeutics. We hereby advocate the need to genotype and perform molecular profiling by multi "omic" analysis on the tumor and stromal cells within the tumor and metastatic sites. Moreover, we suggest that we should examine the adaptive responses to targeted therapeutic agents in mouse models and patients treated with these agents in search of most potent
combinations and mechanisms for resistance. This will be required to affect a cure of this difficult to treat disease.

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