More than the genes, the tumor microenvironment in neuroblastoma

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A B S T R A C T

Neuroblastoma is the second most common solid tumor in children. Since the seminal discovery of the role of amplification of the MYCN oncogene in the pathogenesis of neuroblastoma in the 1980s, much focus has been on the contribution of genetic alterations in the progression of this cancer. However it is now clear that not only genetic events play a role but that the tumor microenvironment (TME) substantially contributes to the biology of neuroblastoma. In this article, we present a comprehensive review of the literature on the contribution of the TME to the ten hallmarks of cancer in neuroblastoma and discuss the mechanisms of communication between neuroblastoma cells and the TME that underlie the influence of the TME on neuroblastoma progression. We end our review by discussing how the knowledge acquired over the last two decades in this field is now leading to new clinical trials targeting the TME.

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Introduction: neuroblastoma, a cancer not solely driven by genetic events

Neuroblastoma is the second most common solid tumor in children and accounts for 8–10% of childhood cancers in the USA and Europe. This cancer derives from neuroepithelial cells that migrate from the neural crest to form the sympathetic nervous system [1]. Children affected with this disease typically present with a posterior, paraspinal mass in the adrenal gland or along the sympathetic chain in the chest or abdomen. In approximately 50% of children the disease presents as a locoregional tumor lacking amplification of the MYCN oncogene, and carries an excellent prognosis (>90% overall survival). However, the other half is considered clinically to be high-risk, either having tumors harboring MYCN amplification or being older (diagnosed in children older than 18 months of age) with metastatic disease irrespective of MYCN amplification. The children with high-risk disease have less than 50% chance of event-free long term survival despite an intensive therapy that combines myeloablative chemotherapy, radiation therapy, progenitor cell transplantation, surgery, isotretinoin and antibody-based immunotherapy [2,3]. The discovery in the early 1980s of a correlation between the amplification of the MYCN oncogene and advanced stage neuroblastoma [4–6] led to the anticipation of other genetic events. About 1–2% of patients with neuroblastoma have a family history of the disease and 2 specific genes, ALK and PHOX2B, have been shown to be mutated (gain of function in the case of ALK, loss of function in the case of PHOX2B) in 80% of the familial cases [7,8]. Genomewide association studies further identified several gene polymorphisms associated with a low but significant risk of neuroblastoma, including BARD1, LMO1 and LIN28B [9]. However a recent genomic analysis of more than 200 neuroblastoma tumors revealed an unexpected low level of recurrent-driver mutations in neuroblastoma. These were mainly activation mutation and amplification of ALK (8% of the cases), activation mutations in PTPN11 (a tyrosine phosphatase), inactivating mutations in chromatin remodeling genes (ATRX and ARID1A) and activating mutations in NRAS in addition to amplification and activation mutations of MYCN [10–12]. A recent analysis in a subgroup of patients with MYCN non-amplified neuroblastoma revealed that metastatic neuroblastomas had higher infiltration of tumor-associated macrophages (TAM) than locoregional tumors. This analysis also revealed that 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. Expression of genes representing TAMs (CD3/CD16/IL-10/FCGR3) in addition to IL-6 receptor (IL-6R) contributed to 25% of the accuracy of a novel 14-gene tumor classification score [13]. Infiltration with Th2-driven macrophages expressing CD163 and CD206 was also recently observed in a subset of high-risk neuroblastoma tumors with deletion of chromosome 11q and high levels of prostaglandin-synthase and elevated levels of PGE2 [14].

In this article we will review the multiple mechanisms by which the TME regulates tumor progression and metastasis in neuroblastoma, focusing on the contribution of innate (TAM, neutrophils, NK,
dendritic cells) and adaptive (T, B, and NKT) immune cells, tumor-associated fibroblasts (TAF), bone marrow-derived mesenchymal stromal cells (MSC), endothelial cells, Schwann cells, and the extracellular matrix (ECM). We will discuss various mechanisms of communication between neuroblastoma cells and the TME used by neuroblastoma cells to “educate” the TME and by TME cells to activate in neuroblastoma signaling pathways affecting their behavior. This review will conclude with a brief discussion on ongoing clinical trials in neuroblastoma patients that target the TME.

Role of the TME in the hallmarks of neuroblastoma

In their article of 2011, Weinberg and Hanahan identified eight critical biological processes controlling cancer progression that they designated “Hallmarks” [15]. These include the ability (1) to sustain proliferative signals, (2) to evade growth-suppressors, (3) to invade and metastasize, (4) to enable replicative immortality, (5) to induce angiogenesis, (6) to resist cell death, (7) to escape immune destruction, and (8) to deregulate cellular metabolism. Genomic instability and tumor promoting inflammation were also recognized as two enabling characteristics. The contribution of the TME to these ten fundamental characteristics of cancer is now evident [16]. It is reviewed in the case of neuroblastoma in this section (Fig. 1).

Ability to sustain proliferative signals

The growth of neuroblastoma cells is regulated by several growth factors that interact with specific receptor tyrosine kinases and non-receptor tyrosine kinases present at their surface. Among the most extensively studied are the neurotrophin receptors – TrkA/NTRK1, TrkB/NTRK2 and TrkC/NTRK3 – that bind to a family of cognate ligands including nerve growth factor (NGF), brain-derived neurotropic factor (BDNF) and neurotrophin-3 (NT3), respectively [17]. These receptors have different effects on proliferation, and whereas the TrkA receptor limits proliferation and promotes differentiation, the TrkB receptor drives proliferation. Activation of these receptors can occur either by an autocrine mechanism by growth factors produced by neuroblastoma cells, by a paracrine mechanism or by autoactivation [18]. Much has been learned on how the TME could participate in the NGFs/NGFR pathway in controlling

Fig. 1. Diagram summarizing the contribution of the cells and ECM in the TME to the ten hallmarks of cancer shown at the center of the wheel. The central graph was reproduced from Hanahan and Weinberg [15].
neuroblastoma cell proliferation from studies on spontaneous regression and differentiation of neuroblastoma tumors [19]. Many neuroblastoma tumors that spontaneously regress or differentiate like ganglioneuroblastoma have a stroma rich in Schwann cells, and although the origin of these cells remains controversial (normal cells that infiltrate the tumor vs. malignant cells sharing the same genetic abnormalities as tumor cells), these cells are an abundant source of NGF and other neurotrophins and cause immature MYCN-amplified (A) and non-amplified (NA) neuroblastoma cell lines to differentiate into benign ganglioneuroblastoma and ganglioneuroma [20–22].

Another cytokine receptor whose function in neuroblastoma has been more recently elucidated is the receptor for interleukin-6 (IL-6R). High expression of this receptor in high-risk MYCN-NA human tumors was reported to be associated with poorer clinical outcomes [13]. Most human neuroblastoma cell lines (MYCN-A and NA) express both the gp130 and gp80 proteins that constitute the IL-6R [23]. They do not express the ligand IL-6 or the soluble agonistic IL-6 receptor (sIL-6R). However, MSC and TAM are an abundant source of IL-6 and sIL-6R in the TME and cooperate to activate signaling transduction and activator of transcription (STAT)3 which promotes proliferation and survival in neuroblastoma cell lines [24]. The ECM also plays a role in controlling the growth of neuroblastoma cells. In most epithelial cancer an increased stiffness in the ECM (deminoplast response) is associated with a stimulatory effect on cell proliferation [25], however it enhances neurogenesis, suppresses cell proliferation and reduces expression of MYCN in an MYCN-A cell line (SK-N-DZ), an effect that is enhanced by retinoic acid [26].

Ability to evade growth-suppressors

The major tumor suppressor pathway in neuroblastoma is p53. However, a striking feature is the low frequency (~2%) of TP53 mutations at diagnosis and even at recurrence [27]. In contrast, alteration of the MDM2 (a p53 inhibitor that promotes p53 degradation) pathway seems to be the mechanism allowing escape from tumor suppression [28]. Evidence that the TME can dysregulate this pathway is currently missing although there is evidence that IL-6 can upregulate MDM2 in transformed and non-transformed cells [29] but this has not been examined in neuroblastoma.

Ability to enable replicative immortality

Like most cancers, neuroblastoma tumors express high levels of telomerase, and such high levels are typically associated with MYCN amplification and poor outcome [30]. Although considered for long to be an intrinsic property of tumor cells, there has been recent evidence demonstrating that telomerase activity in human neuroblastoma cell lines could be controlled by the TME and in particular by inflammatory monocytes/macrophages. It has been shown that exosome-mediated transfer of miR-21 from neuroblastoma cells to monocytes induces the release of miR-155 containing exosomes captured by neuroblastoma cells. Once in neuroblastoma cells, miR-155 directly targets TERT1, an inhibitor of telomerase whose silencing increases telomerase activity promoting chemoresistance in xenograft tumors [31].

Ability to invade and metastasize

The bone marrow, bone and liver are the most common sites of distant metastasis in patients with stage 4 neuroblastoma [32]. Some of the mechanisms involved in bone marrow and bone metastasis have been unraveled. Most human neuroblastoma cell lines derived from patients with high-risk disease (MYCN-A and NA) express the CXCR4 and CXCR7 receptors for the chemokine CXCL12, also known as stromal-derived factor-1 (SDF-1) [33,34]. Expression of CXCR4 in primary tumors also correlates with bone and bone marrow metastasis [35]. CXCR4 expression is associated with highly aggressive undifferentiated tumors, while CXCR7 expression is present in more differentiated and mature tumors. Whereas CXCR4 overexpression in neuroblastoma cells favors dissemination to the liver and the lungs, CXCR7 strongly promotes homing to the adrenal gland and the liver, and co-expression of CXCR4 and CXCR7 receptors significantly and selectively increases neuroblastoma dissemination toward the bone marrow [36,37]. MSC and osteoblasts are a major source of CXCL12/SDF-1 and thus contribute to a microenvironment that promotes bone marrow and bone metastasis [38].

Metastasis in neuroblastoma is associated with a predominant osteolytic process led by activation of osteoclasts [39]. Here also the TME plays a critical role. In bone metastasis, secretion of IL-6 by MSC triggered by neuroblastoma cell lines is a major factor promoting osteoclast activation and bone degradation [40]. The production of insulin-like growth factors (IGF-) 1 and -2 by neuroblastoma cell lines and their release from the bone contribute to this process. IGF acts on type I growth factor receptor IGF-1R expressed by preosteoclasts and promotes their activation [41]. High expression of IGF-1R in neuroblastoma cell lines increases adherence and homing to bone and bone metastasis [42]. Preosteoclasts express the receptor activator of NFkB (RANK) which when interacting with RANK ligand (RANKL) produced by osteoblasts becomes activated and promotes osteoclast maturation and activity [43]. The mechanism of liver metastasis in neuroblastoma is much less understood although gastrin-releasing peptide receptor (GRP-R), expressed in metastatic neuroblastoma cells, may play a role. This receptor activates focal adhesion kinase (FAK), a critical downstream regulator of GRP-R that promotes liver metastasis. FAK expression correlates with GRP-R expression in tumors and cell lines (MYCN-A and NA) [44].

Ability to promote tumor vascularization

Both angiogenesis and vasculogenesis contribute to the vascularization of neuroblastoma tumors. As in most cancers, angiogenesis in neuroblastoma occurs through the production by tumor cells of several angiogenic factors such as vascular endothelial cell growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) whose expression correlates with MYCN amplification and other markers of aggressiveness [45,46]. MYC, being a master regulator of vascular remodeling, has an important paracrine function in angiogenesis [47,48]. MYC knock out in mice is embryonically lethal as a consequence of defective hematopoiesis and vasculogenesis and endogenous suppression of MYC in a pancreatic islet tumor model in mice causes tumor regression through vascular collapse [49]. In neuroblastoma, MYCN upregulates the expression of VEGF [50] and driving MYCN degeneration with inhibitors of PI3K/mTor inhibits tumor progression in TH-MYC transgenic mice, not only through MYCN downregulation but also through a paracrine blockade of angiogenesis [51]. There is also evidence that ALK controls angiogenesis via the production of VEGF as ALK knock-down in human neuroblastoma cells lines transduced with ALK mutant is associated with a marked reduction of VEGF secretion and blood vessel density in xenotransplanted tumors [52]. Although tumor cells are the main source of VEGF, murine and human neuroblastoma cell lines also stimulate the production of VEGF by MSC which promotes osteoblastogenesis through an intracellular mechanism [53]. Schwann cells, which are abundantly present in more differentiated stroma rich tumors, exert an anti-angiogenic function through the production of several inhibitors of angiogenesis such as tissue inhibitor of metalloproteinase 2 (TIMP-2), pigment-derived epithelial factor (PDEF) and secreted protein acidic rich in cysteine (SPARC) [54–56]. Whereas the presence of Schwann cells in tumors is typically associated with decreased vascularization, the
presence of TAF (also inversely related to the presence of Schwann cells) is associated with increased vascularity, thus suggesting that these 2 cell types control the balance of angiogenesis in neuroblastoma [57].

VEGFR2 expressing endothelial progenitor cells (EPC) are recruited to tumors from the bone marrow by a mechanism that is dependent on the production of matrix metalloproteinase-9 (MMP-9) by myeloid cells [58]. MMP-9 promotes the release of soluble c-kit ligand that is necessary for the transition of EPC from a quiescent to a proliferative state in the bone marrow. MMP-9 promotes the recruitment of pericytes into the vasculature [59,60].

**Ability to resist cell death**

Loss of caspase-8 expression by gene silencing or gene deletion is a common feature of aggressive and metastatic neuroblastoma cell lines and patient tumors which allows to avoid programmed cell death [61]. However, when present, caspase-8 has a second function that is regulated by the TME. Paradoxically caspase-8 can promote migration and metastasis in neuroblastoma cell lines (NB 5, 7 and 16) in a manner that is not dependent on its proteolytic and apoptotic activity but that is regulated by contact with the ECM. Upon integrin ligation to ECM proteins, caspase-8 forms a complex with FAK, calpain2/CPN2 and calpastatin which, by disrupting CPN2/calpastatin interaction, activates CPN2. Activation of CPN2 enhances the cleavage of focal adhesion substrates and migration of MYCN-A and MYCN-NA human neuroblastoma cell lines in vitro [62]. There is also evidence that the TME promotes survival in neuroblastoma cell lines by upregulating anti-apoptotic proteins. For example, activation of STAT3 by IL-6 and sIL-6R produced by MSC and TAM induces the expression of several pro-survival proteins like survivin, Bcl-2 and anti-apoptotic Bcl-XL that cause resistance to chemotherapeutic agents [24]. In contrast, IL-12 produced by Th1-driven TAM increases apoptosis in orthotopic murine neuroblastoma tumor models via tumor necrosis factor (TNF)-α and interferon (IFN)-γ which increase the expression of pro-apoptotic proteins, inhibit Akt phosphorylation and activate Bid [63].

**Ability to escape the immune system**

Neuroblastoma cells have developed multiple mechanisms to escape the immune system. Cell lines typically have low levels of expression of peptide presenting HLA class I molecules, which impairs target peptide recognition by cytotoxic T cells (CTL) [64–66]. The expression of major histocompatibility complex (MHC) I in neuroblastoma cell lines is controlled by the TME. For example, INF-γ produced by NK, NKT and T cells increases the expression of MHC I [65,67] and activation of NTRK1/TrkA upon ligation with NGF leads to an increased immunogenicity by overexpression of MHC I proteins [68], whereas MYCN amplification causes a downregulation of MHC I [69]. Neuroblastoma cells also escape immune attack by CTL and NK cells by producing soluble MHC-I related chain (sMICA) and by expressing CD276/B7-H3, a co-stimulatory protein, which inhibit activation of NK cells and T cells via interaction with their receptors. Downregulation of the NGK2D ligands observed in human primary tumors and cell lines also contributes to immune evasion [70,71]. Neuroblastoma cell lines (6 out of 12) express low levels of HLA-G but induce monocytes to release soluble HLA-G, a protein that induces immune tolerance during pregnancy and is a ligand for the NK cell inhibitory receptor KIR2DL4 [72]. TAM further contributes to this immunosuppressive environment because they are a source of secreted immunosuppressive cytokines including IL-6, IL-10, and transforming growth factor (TGF)β1. Despite this suppressive environment, NK cells, which in contrast to CTL do not depend on the expression of MHC I proteins on tumor cells, may be more effective although they still can be inhibited. Type 1 NKT cells do not depend on CD1d expression in neuroblastoma cells to be active in tumors [73]. When activated by IL-2, NK cells secrete IFN-γ and become cytotoxic via perforin-dependent and independent mechanisms [74]. Activated NKT cells secrete IL-2 which in turn activates NK cells [73] and eliminates TAM [75].

There is evidence that MYCN has a paracrine regulatory role on immune cells in the TME. For example, MYCN downregulates CCL2/MCP-1 (macrophage chemoattractant protein) which attracts NKT cells in tumors [76,77]. The contribution of immune check points such as PD-1/PD-L1 and CTLA-4/B7-1 to immunosuppression has just begun to be explored. In murine neuroblastoma models mAb targeting 4-IBB, CD40 and CTLA-4 induces tumor regression [78]. There is also pre-clinical evidence that PDL-1 blockade potentiates immunogenicity of human neuroblastoma cell lines [79].

**Ability to deregulate cellular metabolism**

Many cancers preferentially meet their energy requirements through the glycolytic pathway rather than via the more efficient oxidative phosphorylation pathway (the Warburg effect). Human neuroblastoma cell lines (MYCN-A and NA) in vitro preferentially use the glycolytic pathway in a way that is not dependent on MYCN [80]. The contribution of the TME to this effect has not been well explored and is thus poorly understood. There is some suggestion that dietary restriction leading toward low glucose concentration may inhibit the Warburg effect and increase cytotoxicity in neuroblastoma cell lines [81].

**Genomic instability**

Genomic instability has been extensively studied in neuroblastoma as discussed in our introduction. There is however little evidence so far that genomic instability can be affected by the TME. Considering that telomere-dependent chromosomal instability is highly prevalent in aggressive MYCN-NA neuroblastoma [82], it is conceivable that it could be affected by regulators of telomerase such as TERT1, whose expression in neuroblastoma cell lines has been shown to be dependent on an interactive loop between neuroblastoma cells and TAM [31].

**Induction of a pro-tumorigenic inflammation**

Innate immune cells such as monocytes/macrophages and neutrophils can create in neuroblastoma tumors an inflammatory environment that affects tumor progression and metastasis. There is evidence that neuroblastoma cells educate TAM toward a Th2-driven phenotype that supports their progression. The presence of macrophages in neuroblastoma tumors is an indicator of poor outcome and more aggressive disease in MYCN-NA high risk tumors [13,14], and blocking macrophage stimulatory factor (CSF-1) in CSF-1 negative xenotransplanted neuroblastoma tumors extends survival in tumor-bearing mice [83]. The presence of these macrophages is also associated with elevated levels of PGE2 and the presence of cancer-associated fibroblasts [14]. In contrast, neutrophils can be cytotoxic and inhibit the growth of human neuroblastoma cell lines (GD2 positive and negative) when in the presence of a chimeric anti-GD2 antibody but promote tumor cell growth in the absence of such antibody or if GD2 is not expressed [84,85].

There is thus growing evidence that the TME plays a regulatory role in neuroblastoma progression. The ultimate effect however is the result of dynamic and complex interactions between neuroblastoma cells and TME cells, leading toward an environment that is pro- or anti-tumorigenic. In the next section, we will review several mechanisms of communication between neuroblastoma cells and TME cells and the ECM that regulate these interactions (Fig. 2).
Mechanisms of communication between neuroblastoma cells and TME cells

The reciprocal communication between neuroblastoma cells and the TME is based on 2 fundamental types of mechanism, (i) adhesion-dependent mechanisms that involve a contact between tumor cells and TME cells (cell–cell contact) or between tumor cells and components of the ECM (cell–ECM contact); and (ii) adhesion-independent mechanisms through the production of soluble products.

Adhesion-dependent mechanisms

Cell–TME contact

Neuroblastoma cell lines (SK-N-SH and LAN-1) are able to interact directly with endothelial cells [86] and leukocytes [87] as they express cell adhesion molecules (CAM) of the IgG superfamily like VCAM-1 and ICAM-1 which promote cell migration and metastasis. The expression of VCAM-1 and ICAM-1 in neuroblastoma cell lines (SK-N-SH and SK-N-MC) is upregulated by inflammatory cytokines, such as TNF-α and IFN-γ [88].

Neuroblastoma cells express CD56/NCAM which mediates their interaction with CD56/NCAM positive NK cells. It has been suggested that such interaction augments tumor cell lysis by NK cells but the importance of such function is not entirely clear [89–91].

Cell–ECM contact

Neuroblastoma cells interact with the ECM through the expression of several integrins. The effect that integrins have on neuroblastoma progression depends on the type of integrin expressed and the type of ECM protein involved. For example, the integrin α5β1 that mediates adhesion to collagen and fibronectin has an inhibitory function on migration. In vitro substrate adherent (S-type) neuroblastoma cells with high levels of α5β1 are less migratory and tumorigenic, whereas neuroblastic (N-type) neuroblastoma cells with low levels of integrin α5β1 do not attach to fibronectin and collagen IV, and are more migratory and tumorigenic. Consistently, blocking of α5β1 integrin in S-type cells promotes migration and the overexpression of α5β1 integrin into N-type cells increases their attachment to fibronectin and collagen IV and inhibits migration [92,93]. IGF-1R stimulation decreases β1-integrin promoting cell migration and invasion [94]. There is also a reverse correlation between β1 integrin and MYCN expression and progression in neuroblastoma cell lines and xenografts. Over-expression of MYCN in transfected SK-N-SH cells downregulates the expression of β1 integrin [95,96]. Consistently, human neuroblastoma
tumors with good prognosis have a high expression of α5β1 integrin, whereas in tumors that originate from patients with metastatic disease there is a lack of α5β1 integrin expression and high level of αvβ3 integrin [97].

Other integrins promote migration. This is the case of α3β1 and α1β1 integrins that interact with laminin promoting migration but also neurometastoutgrowth in the N-type SH-SY5Y human neuroblastoma cell line in a manner that is dependent on O-mannosyl-linked glycosylation events [98,99]. Binding of integrins to ECM proteins activates FAK and Src [100]. FAK-Src activation stimulates neuroblastoma cell motility but also recruits several adapter proteins such as talin and paxillin as well as the protease CPN2/ calpain [101]. As previously discussed, caspase-8 is also recruited in this complex, is inactivated by Src-dependent phosphorylation of Tyr380 and activates CPN2, which promotes cell migration in apoptosis resistant cells [62,102,103]. Binding of integrins to fibronectin in mouse neuroblastoma cell lines also promotes the activation of cyclin E and cell proliferation [104]. α4 integrin is more frequently expressed in MYCN-NA human neuroblastoma cell lines and is selectively associated with a poorer outcome in MYCN-NA tumors. It interacts with an alternatively spliced variant of fibronectin, as well as with VCAM-1 promoting migration in vitro and metastasis in vivo [105].

CD44, a transmembrane glycoprotein that mediates cell adhesion to hyaluronan, is expressed by neuroblastoma cells. Its expression correlates with a non-metastatic phenotype and is commonly seen in MYCN-NA neuroblastoma tumors [106,107]. MYCN-A neuroblastoma cell lines (SHEP and ACN) do not express CD44 or express a non-functional variant of CD44 that does not bind to hyaluronan [108].

Disialoganglioside (GD2), a surface glycolipid present on neurons, peripheral nerve fibers, and skin melanocytes, is highly expressed in primary neuroblastoma tumors and is a target for immunotherapy [109]. GD2 facilitates the attachment of tumor cells to ECM proteins such as collagen, vitronectin, laminin, and fibronectin [110,111], which is blocked by the anti-GD2 mAb 14G2a leading to cell death [112].

Adhesion-independent mechanisms

The reciprocal communication between neuroblastoma cells and TME cells also involves soluble products providing paracrine and distant mechanisms of communication. Whereas much of the focus has been on the contribution of proteins there has been recent evidence that extracellular vesicles (EV) carrying not only proteins but also DNA and RNA could play an important role [31]. In this section, we will focus on some of the major chemokines, cytokines and growth factors specifically involved in paracrine interaction between neuroblastoma cells and stromal cells, illustrating how they are used by TME cells to activate specific signaling pathways in tumor cells and how they are used by tumor cells to “educate” the TME.

Axes of communication from TME cells to tumor cells

CCL12/CXCR4/Akt axis. As previously discussed, the chemokine CCL12/SDF-1, produced by MSC and osteoblasts in the bone marrow niche, plays an important role in the establishment of distant metastasis by attracting neuroblastoma cells that express CXCR4 and CXCR7 [38]. Interaction between CCL12/SDF-1 with its receptor CXCR4 induces cell migration, proliferation and survival in an Akt-dependent mechanism that is inhibited by dipetidyl peptidase IV, a cell surface serine protease present in some neuroblastoma cell lines [113].

IL-6/IL-6R/STAT3 axis. Neuroblastoma cell lines do not make IL-6, but upregulate its expression by MSC and TAM. In addition to activating osteoclasts and promoting osteolytic bone metastasis [40], IL-6 binding to its receptor IL-6R expressed by neuroblastoma cell lines exerts a pro-tumorigenic effect on tumor cells, enhancing growth, survival and drug resistance in a STAT3-dependent mechanism [23,24].

VEGF/VEGFR/Akt and ERK1/2 axis. Among the growth factors involved in the interaction between neuroblastoma cells and stromal cells is VEGF. Neuroblastoma cell lines, in particular MYCN-A, produce large amounts of VEGF but also stimulate its production in TME cells. Several studies reported the expression of VEGF receptors, primarily neuropilin-1 and 2, in neuroblastoma cell lines and some expression of VEGFR1 and VEGFR2 mRNA in state III tumors [114–117]. VEGF promotes cell survival of neuroblastoma cells via the PI3K/Akt pathway [118] and resistance to etoposide by activating ERK1/2 and upregulating BCL-2 [119].

IGF-1/IGFR/ERK1/2 and PI3K/Akt axis. IGF-1 is secreted by many cells including MSC [120] and endothelial cells [121]. It is also released from the bone matrix during osteolysis [122]. N-type neuroblastoma cell lines express the IGF-1 receptor (IGF-1R) [42]. Binding of IGF-1 to its receptor activates ERK1/2 and PI3K/Akt and promotes cell migration [54,123–125] and homing in the bone marrow [42]. It also stimulates neuroblastoma cell survival in SHEP cells and protects them from apoptosis through the upregulation of BCL-2 and inhibition of caspase-3 [126]. This effect could be particularly significant in MYCN-A neuroblastoma cell lines since there is an increased expression of IGF-1R in MYCN-transfected cells [127].

TGF-β/TGFBR/SMAD axis. TGF-β is secreted by many TME cells such as MSC and TAM, and is also released in the bone environment upon bone degradation [128–131]. In addition to being a potent inhibitor of the immune system, TGF-β directly acts on neuroblastoma cells by affecting proliferation and differentiation in a way that depends on the receptors expressed. Neuroblastoma cells express various levels of TGFBR (I, II and III) with expression of TGFBRII and III being inversely correlated with poor outcome. Consistently, TGF-β inhibits cell proliferation in human neuroblastoma cell lines expressing type II and III receptors [131] and overexpression of TGFBRII suppresses tumorigenesis [132]. TGFBRII promotes differentiation and suppresses neuroblastoma proliferation whereas TGFBR1 only promotes differentiation [132]. Furthermore, evidence that PI3K/Akt negatively regulates SMAD2/4 signaling in neuroblastoma suggests that an imbalance between PI3K/Akt and TGF-β/SMAD signaling is a mechanism promoting tumorigenesis in neuroblastoma [133].

PDGF/PDGFR-β and PI3K/Akt and ERK1/2 axis. PDGF is secreted by endothelial cells [134], preosteoclasts [135], and can interact with neuroblastoma cell lines that express PDGFR. PDGFR activation stimulates migration, invasion and proliferation in PI3K and ERK dependent mechanisms [136].

Soluble products secreted by neuroblastoma cells that “educate” TME cells

Much less is known of the soluble products released by neuroblastoma cells that interact and educate TME cells. The contribution of neuroblastoma-derived VEGF to the autocrine (neuroblastoma cells) and paracrine (endothelial cells, MSC) activation of signaling pathways promoting tumor cell growth and angiogenesis is well known and has been discussed previously. Neuroblastoma cells secrete MCP-1/CCL2 that interacts with the CCR2 receptor expressed by type I NKT cells which attracts them in the tumor. The expression of MCP-1 by neuroblastoma cells is downregulated by MYCN thus preventing the recruitment of type I NKT cells and contributing to immune escape [76,6].

Human neuroblastoma cell lines produce galectin-3 binding protein (Gal-3BP), a sialoglycoprotein also present in neuroblastoma
tumors that interact with galectin-3 present in TME cells such as TAM and MSC. Gal-3BP interaction with Gal-3 induces the transcriptional activation of IL-6 in a RAS/MEK/ERK-dependent mechanism [137,138].

There is also recent evidence that the secretion of extracellular vesicles including exosomes by tumor cells is a mechanism used to educate TME cells and, for example, to induce the establishment of a pre-metastatic niche [139]. These vesicles contain a large variety of proteins, lipids, metabolites and nucleic acids including regulatory miRNAs that act as shuttles mediating the communication between tumor cells and stromal cells in both directions. The protein content of the cargo of exosomes from several neuroblastoma cells includes, in addition to the exosomal markers (CD63, CD9, CD81, Hsp70, Hsp 90, Alix), GD2 disialoganglioside and proteins involved in tumor progression such as CD147, a multifunctional protein involved in invasion and metastasis, and CD276/B7-H3, an immune checkpoint protein that protects neuroblastoma cells from attack by NK cells [140]. The profile of miR content of exosome-like particles from two MYCN-A neuroblastoma cell lines was also recently reported showing that the exosomal miRNAs are associated with a range of cellular and molecular functions related to cell growth, cancer progression and drug resistance [31,141].

Therapeutic consideration

With a better knowledge of the contribution of the TME to neuroblastoma progression and of its mechanisms, clinical trials testing agents targeting the TME have been initiated. These trials follow three main strategies including (1) Direct targeting of TME cells that contribute to a pro-tumorigenic environment, (2) Targeting signaling pathways activated by the TME in tumor cells or in TME cells (or both), and (3) Immunotherapy. A complete discussion of these strategies is beyond the scope of this review article but a few illustrative examples of ongoing Phase I/II clinical trials are given in Table 1.

Strategies targeting TME cells

Targeting osteoclasts with bisphosphonates like zoledronic acid has been tested in patients with bone metastasis and the results of a Phase I trial completed by the New Advances in Neuroblastoma Therapy (NANT) consortium demonstrated the safety and efficacy of zoledronic acid [142]. This agent is now tested with cyclophosphamide in a Phase I clinical trial by Baylor College of Medicine (NCT00206388) and in combination with Interleukin-2 in young patients with recurrent or refractory neuroblastoma (N2001-03: CEP-701 in Treating Young Patients With Recurrent or Refractory Neuroblastoma — NANT). This agent is now tested with cyclophosphamide and zoledronic acid in combination with Interleukin-2 in young adults with neuroblastoma (NANT). The profile of miR content of exosome-like particles from two MYCN-A neuroblastoma cell lines was also recently reported showing that the exosomal miRNAs are associated with a range of cellular and molecular functions related to cell growth, cancer progression and drug resistance [31,141].

Table 1
Registered Phase I/II clinical trials in neuroblastoma that target the TME. The list was generated from data on clinicaltrials.gov.

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<th>Category</th>
<th>Pathway(s)</th>
<th>Molecular target(s)</th>
<th>Study</th>
<th>Phase</th>
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<td>Tumor microenvironment</td>
<td>Osteoclast survival</td>
<td>Farnesyl pyrophosphate synthase</td>
<td>Zoledronic Acid (ZOMETA) With Cyclophosphamide With Neuroblastoma and Cortical Bone Involvement</td>
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refractory neuroblastoma by University of Alabama at Birmingham (NCT01404702).

Targeting endothelial cells in neuroblastoma has also been tested and a Phase I trial with bevacizumab, cyclophosphamide and zoledronic acid in patients with recurrent or refractory high-risk neuroblastoma has been recently completed by the NANT consortium (NCT00885326).

Strategies targeting pathways activated by the TME

These strategies typically combine a small molecule inhibitor of a signaling pathway with standard therapy. For example, sorafenib, a broad spectrum kinase inhibitor with pre-clinical activity in neuroblastoma [143,144], is presently tested in Phase I in combination with cyclophosphamide and topotecan in patients with relapsed and refractory neuroblastoma by NANT (NCT02298348). ZD6474, an inhibitor of VEGFR and EGFR, is also tested in Phase I alone and in combination with retinoic acid in neuroblastoma by M.D. Anderson Cancer Center (NCT00533169). Lestaurtinib (CEP-701), an inhibitor of Jak2, TrkA, TrkB and TrkC, is actually tested in Phase I in young patients with recurrent or refractory high-risk neuroblastoma by NANT (NCT00084422). R(+)XK469, an inhibitor of topoisomerase II-β and MEK/ERK signaling kinases, is used in treating patients with advanced neuroblastoma in Phase I by NCI (NCT00028522). Temsirolimus, a rapamycin analog inhibitor of the Akt pathway, is presently tested in combination with irinotecan and temozolomide in patients with relapsed pediatric solid tumors including neuroblastoma [145] by the NCI (NCT01767194). SF1126, a dual PI3K/Akt and mTOR inhibitor, is tested in patients with refractory/recurrent neuroblastoma (NCT02337309). In addition, a combination of Rapamycin, mTOR inhibitor, with dasatinib is currently tested in patients with relapsed or refractory high-risk Neuroblastoma by the University of Regensburg (NCT01467986).

Immunotherapies

Whereas the human-mouse chimeric 14:18 anti-GD2 monoclonal antibody is now part of the standard therapy of neuroblastoma [3], other anti-GD2 antibodies are tested. The monoclonal antibody 3F8 is currently tested in a Phase II trial in combination with sargramostim in patients with neuroblastoma (NCT00072358). In addition, another clinical trial sponsored by Memorial Sloan Kettering Cancer Center is testing the antibody 3F8 in combination with GM-CSF (NCT00450307) [146]. The same antibody 3F8 coated in T cells to promote cytotoxicity is also the subject of a Phase I trial. Activated T cells armed with a GD2 bisspecific antibody are tested in Phase I in children and young adults with neuroblastoma at the Barbara Ann Karmanos Cancer Institute (NCT02173993). New strategies using immunomodulators like lenalidomide [147], vaccine therapy and engineered T cells and NK cells are planned, often in combination with anti-GD2 immunotherapy, by several groups [148–150]. This is an area of intensive investigation.

Conclusion

After much focus on genetic aspects of neuroblastoma biology in the late 1990s and early 2000s, it is now clear that neuroblastoma is truly a disease of the seed and the soil, and that the TME significantly contributes in a favorable or unfavorable way to its progression. The observations summarized in this article raised the interesting and unexplored question of the potential interaction between genetic driver events in neuroblastoma and their influence through paracrine mechanisms on the TME. In other words, do genetic alterations in the seeds have the ability to influence the soil in a way that is critical for their development and survival? We have reviewed some evidence, for example, that MYCN and ALK could exert a paracrine control on angiogenesis. Thus targeting MYCN will have a dual effect on the seed and the soil.

As we better appreciate the tremendous level of complexity in the interactions between neuroblastoma cells and the TME, the challenge will be to distinguish interactions that play a more dominant role affecting both tumor and TME cells from those that have a less important function, to identify effective agents for these pathways and to develop biomarkers to monitor their effects. The study of the TME in neuroblastoma will continue to be an important field of investigation that lead to new therapies.

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Conflict of interest

None.

References


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