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PD-L1, Inflammation, non-coding RNAs, and Neuroblastoma: Immuno-oncology Perspective

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Abstract

Neuroblastoma is the most common pediatric solid tumor of neural crest origin. The current treatment options for neuroblastoma produce severe side effects. Programmed death-ligand 1 (PD-L1), chronic inflammation, and non-coding RNAs are known to play a significant role in the pathogenesis of neuroblastoma. Cancer cells and the surrounding cells in the tumor microenvironment express PD-L1. Programmed death-1 (PD-1) is a co-receptor expressed predominantly by T cells. The binding of PD-1 to its ligands, PD-L1 or PD-L2, is vital for the physiologic regulation of the immune system. Chronic inflammation is involved in the recruitment of leukocytes, production of cytokines and chemokines that in turn, lead to survival, metastasis, and angiogenesis in neuroblastoma tumors. The miRNAs and long non-coding (lnc) RNAs have emerged as a novel class of non-coding RNAs that can regulate neuroblastoma associated cell-signaling pathways. The dysregulation of PD-1/PD-L1, inflammatory pathways, lncRNAs, and miRNAs have been reported in clinical and experimental samples of neuroblastoma. These signaling molecules are currently being evaluated for their potential as the biomarker and

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therapeutic targets in the management of neuroblastoma. A monoclonal antibody called dinutuximab (Unituxin) that attaches to a carbohydrate molecule GD2, on the surface of many neuroblastoma cells, is being used as an immunotherapy drug for neuroblastoma treatment. Atezolizumab (Tecentriq), an engineered monoclonal antibody against PD-L1, are currently in clinical trial for neuroblastoma patients. The lncRNA/miRNA-based therapeutics is being developed to deliver tumor suppressor lncRNAs/miRNAs or silencing of oncogenic lncRNAs/ miRNAs. The focus of this review is to discuss the current knowledge on the immune checkpoint molecules, PD-1/PD-L1 signaling, inflammation, and non-coding RNAs in neuroblastoma.

Keywords

Neuroblastoma; PD-L1; Inflammation; non-coding RNAs; Immuno-Therapy

1. Introduction

Neuroblastoma is the most common childhood cancers that originate from neuroblast cells. During development of a fetus, neuroblasts are transformed into nerve cells. However, mutations in the small portion of immature neuroblasts can lead to neuroblastoma [1]. According to one estimate, the incidence of neuroblastoma in the United States is 1 per 100,000 children and approximately 700 children (younger than 15 years) suffer from this disease each year [2]. The majority of neuroblastoma patients are diagnosed at an advanced stage. Although surgery, radiation, and chemotherapy are common treatment options, neuroblastoma cells often develop resistance mechanisms and the disease relapses [3]. The patients with relapsed neuroblastoma are highly incurable [4]. Neuroblastoma has a poor prognosis especially when diagnosed in an advanced stage. Thus, improved detection and therapeutic methods for the diagnosis, prognosis, and therapy are required.

The immune checkpoint inhibition molecules such as programmed death protein-1 (PD-1) and its ligands (PD-L1, PD-L2), inflammatory molecules, and non-coding RNAs are known to play a significant role in the neuroblastoma pathogenesis. PD-1 is the major immune checkpoint receptor expressed on activated monocytes, B cells, T cells, dendritic cells (DCs), and natural killer T cells in humans and mice [5–8]. It plays a significant role in cell adhesion, proliferation, and cytokine signaling. It can also promote self-tolerance by suppressing T cell function [9, 10]. The inflammatory molecules (cytokines, chemokines) and transcription factors (NF-κB, STAT3) are dysregulated in many tumor types including neuroblastoma [11–13]. These inflammatory molecules play a role in modulating immunosurveillance, promoting angiogenesis and recruiting leukocytes to neuroblastoma cells [14, 15]. Non-tumor cells and factors such as tumor-associated macrophages (TAMs) and macrophage migration inhibitory factor (MIF) are also the major mediators of inflammation.

It is now clear that ~98% of the human genome accounts for non-coding sequences [16]. Furthermore, ~90% of these non-coding sequences are transcribed to produce a large number of non-coding RNAs [17–20]. There are two major classes of non-coding RNAs: microRNAs (miRNAs, 18–22 nucleotides) and long non-coding RNAs (lncRNAs, 200

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nucleotides). Although miRNAs are well characterized, lncRNAs are relatively new. During recent years, lncRNAs have been implicated in regulating cellular functions and disease processes including neuroblastoma [21–23]. Because of specificity and ease in detection, lncRNAs can be used as a biomarker and therapeutic target [24–27]. miRNAs can also regulate different physiological and pathological processes including inflammation and cancer [28–30]. miRNAs are known to negatively regulate protein coding genes and the expression of other non-coding transcripts. miRNAs are involved in the post-transcriptional modulation of multiple genes by base-pairing to target mRNAs [31]. The binding of miRNAs to the 3'untranslated region (UTR) of mRNAs usually leads to the degradation or translational repression [32]. Accumulating evidence suggests that miRNAs play a significant role in the pathogenesis of neuroblastoma [2], and thus could be used for the diagnosis and prognosis of disease [33–37].

In the following sections, we discuss the role of immune checkpoint molecules, inflammatory molecules, PD-1/PD-L1 signaling, miRNAs, and lncRNAs in neuroblastoma pathogenesis. We also discuss the clinical implications of these molecules in neuroblastoma. We provide evidence that these molecules can be used as a biomarker and therapeutic target for neuroblastoma (Figure 1).

2. Immune Checkpoint molecules

T-lymphocytes are primary effector cells of the adaptive immune response against cancer, which includes helper T cells and cytotoxic T cells. The cytotoxic T cells directly attack the tumor cells and helper T cells to propagate the anti-tumor immune response in the immune system. These cells play an important role in the recognition of tumor antigens from the major histocompatibility complex (MHC) receptors. The interaction of T cell receptor and tumor antigen MHC complex on antigen-presenting cells is called as T-cell priming/ activation [38]. The priming of T-cells is firmly coordinated by immune checkpoint molecules. The most common immune checkpoint molecules are cytotoxic lymphocyte–associated protein 4 (CTLA-4) and PD-L1. CTLA-4 and PD-L1 are over expressed on most cancer types and are known to inhibit the T cell function [39].

Lymphocyte-activation gene 3 (LAG 3) are MHC class 2 ligand immunecheck point molecules, expressed in dendritic cells (DCs) and tumor-infiltrating macrophages. It augments regulatory T cells (T reg) and impede the CD 8 effector T cell to allow the cancer cell immune escape [40]. T cell membrane protein 3 (Tim-3) is an immune checkpoint receptor molecules found in natural killer T cells, macrophages and DCs [41]. It is highly expressed in many cancers including liver, lung and melanoma tumors. BTLA is a B- and T-lymphocyte attenuator protein immune checkpoint molecules, expressed in activated T-helper cells. It is associated with a ligand in herpes virus entry mediator to mediate the signal recipient cancer cells. It is expressed on the surface of melanoma cancer cells and alleviates IL-2 to impair the activation of T cells [42]. Considering the fact that immune checkpoint molecules play major role in cancer therapy, a number of inhibitors are being evaluated by clinical trials [43].

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2.1. PD-1 and PD-L1 Signaling

2.1.1. Programmed Death-1 Protein (PD-1)—PD-1 also known as cluster of differentiation 279 (CD279), is a cell surface receptor that belongs to immunoglobulin gene super family [44]. PD-1 was isolated in 1992 using subtractive hybridization. It is expressed on activated monocytes, B cells, dendritic cells, NK cells, natural killer T (NKT) cells, T cells, regulatory T cells (Treg), and exhausted T cells [5, 45, 46]. PD-1 plays a crucial role in lowering the immune system through suppression of T-cell function and up-regulation of Tregs, which in turn, reduces autoimmunity and promotes self-tolerance [9, 10]. Under normal physiological conditions, PD-1 is known to interact with two ligands, PD-L1 and PD-L2; these ligands share 37% sequence homology [47–49]. The expression of PD-L1 and PDL2 is increased when cancer cells are attacked by the immune system, leading to the suppression of T cells and immune escape [50].

2.1.2. Programmed Death Ligand-1 (PD-L1)—PD-L1 is a 40-kDa type 1 transmembrane protein that suppresses the immune system during physiological and pathological events (Dong et al.,). PD-L1 is constitutively expressed on antigen presenting cells, non-lymphoid organs and non-hematopoietic cells such as heart, lung, placenta, and liver. PD-L1 is also expressed by a variety of cancer cells and by tumor-infiltrating immune cells including dendritic cells and macrophages [51]. It can be induced by various pro-inflammatory cytokines like IFN-γ, TNF-α, VEGF, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10. PD-L1 can also be induced by toll like receptors (TLRs), interferon regulatory factor 1 (IRF1), STAT1, STAT3, and hypoxia-inducible factor-1alpha (HIF-1α) [52]. Up-regulation of PD-L1 in tumor cells facilitates immune suppression in the tumor microenvironment [53]. Upon epidermal growth factor receptor (EGFR) stimulation, mature PD-L1 undergoes glycosylation, which leads to its stabilization [54]. In the absence of glycosylation, PD-L1 undergoes GSK-3β mediated phosphorylation which triggers its K48-ubiquitination and subsequent degradation [54]. PD-L1 can also be stabilized by COP9 signalosome (CSN5) mediated deubiquitination [55]. Furthermore, nuclear factor (NF)-κB p65 can directly regulate CSN5 expression by binding to its promoter leading to PD-L1 stabilization and immune suppression [55]. The IFN-γ-induced PD-L1 expression is dependent on the NF-κB signaling [56]. In human glioma cells, dysfunction in PTEN and activation of PI3K is associated with increased expression of PD-L1 [57]. PI3K can also increase the translation of PD-L1 mRNA [47]. These studies clearly demonstrated that PD-1 and PD-L1 contribute to cancer pathogenesis and thus could be used as biomarkers and therapeutic targets.

2.1.3. Diagnostic and Prognostic Significance of PD-1/PD-L1—Several lines of evidence have established the diagnostic and prognostic potential of PD-1/PD-L1 in neuroblastoma. For example, one study was aimed to characterize the PD-L1 expression and tumor-associated immune cells (TAICs) (lymphocytes and macrophages) in common pediatric cancers [58]. The whole slide sections and tissue microarrays were evaluated by immunohistochemistry for PD-L1 expression and the presence of TAICs. TAICs were also screened for PD-L1 expression. Thirty-nine of 451 evaluable tumors (9%) expressed PD-L1 in at least 1% of tumor cells. The common cancer types observed in these pediatric cancer patients were Burkitt lymphoma, glioblastoma multi-forms, and neuroblastoma. PD-L1

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staining was associated with inferior survival in neuroblastoma patients. Furthermore, 74% of tumors contained lymphocytes and/or macrophages. The macrophages were more likely to be identified in PD-L1-positive versus PD-L1-negative tumors. The authors of this study concluded that a subset of diagnostic pediatric cancers exhibit PD-L1 expression, whereas a much larger fraction show infiltration by tumor-associated lymphocytes. Furthermore, PD-L1 expression could be used as a biomarker for poor outcome in neuroblastoma patients. However, more studies are required to define the predictive nature of PD-L1 expression in neuroblastoma both at diagnosis and after chemotherapy.

N-Myc (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-Derived Homolog) is a proto-oncogene encoded by MYCN that belongs to the Myc family of DNA binding basic helix-loop-helix leucine zipper proteins. Previous studies have demonstrated the association of MYCN amplification and N-Myc over-expression with several cancer types, most notably neuroblastoma [59–62]. MYCN is used as a biomarker to stratify and to assess neuroblastoma patients [63, 64]. MYC and MYCN are known to regulate PD-L1 in neuroblastoma [64, 65]. The functional inhibition of MYC/MYCN is known to suppress PD-L1 expression [64]. Furthermore, MYC can initiate and maintain tumorigenesis through the modulation of immune regulatory molecules [65]. Toll-like receptor 3 (TLR3) can also enhance PD-L1 and major histocompatibility complex (MHC) class I expression in neuroblastoma cells [66]. Expression of TLR3 can also serve to predict favorable prognosis in neuroblastoma [67]. Treatment of neuroblastoma cell lines with TLR3 or interferon-γ can significantly up-regulate PD-L1 and MHC class I [68].

A study was sought to evaluate the expression of PD-L1 and HLA class I on neuroblastoma cells, and PD-1 and lymphocyte activation gene 3 (LAG 3) on tumor-infiltrating lymphocytes to examine if neuroblastoma patients may benefit from therapies targeting immune checkpoint molecules [64]. The expression of PD-L1, HLA class I, PD-1 and LAG3 was assessed in 77 neuroblastoma specimens by *in situ* immunohistochemistry (IHC). These patients were characterized by tumor-infiltrating T-cell density that correlated with clinical outcome. A data set of 477 human primary neuroblastomas from Gene Expression Omnibus (GEO) and array expression databases was explored for PD-L1, MYC, and MYCN correlation. The combination of PD-L1 and HLA class I tumor cell density was found to be a prognostic biomarker for predicting overall survival in neuroblastoma patients. The abundance of PD-L1 transcript correlated with MYC expression in primary neuroblastoma. It was concluded that the combination of PD-L1 and HLA class I could be a novel prognostic biomarker for neuroblastoma.

Dondero and colleagues analyzed the effect of constitutive and inducible expression of PD-Ls in human neuroblastoma cell lines, *ex vivo* isolated neuroblasts, and lymphocytes [69]. A combination of PD-L1 and human leucocyte antigen (HLA) class I tumor cell density was identified as a prognostic biomarker for predicting overall survival in neuroblastoma patients. In another study, the infiltrating T cells were found to possess prognostic value greater than the currently used methods [70].

Relapsed/refractory neuroblastoma (rNB) after traditional chemotherapy is highly incurable. A recent clinical trial from USA (https://clinicaltrials.gov/ct2/show/study/NCT02868268) is

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recruiting rNB patients. This clinical trial is utilizing the multi-institutional infrastructure and Translational Genomics Research Institute GEM sequencing platform. The aim of the trial is to identify subgroups of rNB patients with potentially targetable genetic (ALK, MAPK, metabolic-related genes) and/or immunologic (tumor-associated macrophage infiltration and/or PD-L1 expression) biomarkers in rNB. It is expected that understanding the genetic and immunologic landscape of rNB will help in devising novel therapies for these patients.

2.1.4. Therapeutic Potential of PD-1/PD-L1—PD-1 inhibitors block the interaction of PD-L1 and PD-L2 with PD-1 on T cells and increase T cell proliferation and function [71]. The molecular basis for PD-1 and PD-L1 based therapy is presented in Figure 2. In fact, some inhibitors have been developed against PD-1/PD-Ls. For example, opdivo (nivolumab) and keytruda (pembrolizumab) are PD-1 inhibitors approved by FDA for advanced melanoma and non-small cell lung cancer (NSCLC).

PD-L1 antibodies act by blocking the interaction of PD-L1 with PD-1 without affecting PD-L2/PD-1 interaction. Because the PD-1/PD-L2 pathway plays a role in peripheral tolerance, the specificity of PD-L1 antibodies may help to decrease toxicity. Some monoclonal antibodies developed against PD-L1 are BMS-986559 (MDX-1105), MPDL3280A, and MEDI4736. These antibodies are currently under evaluation in clinical trials for advanced malignancy (http://www.clinicaltrial.org/). However, the efficacy of these antibodies against neuroblastoma is yet to be determined. To our knowledge, dinutuximab (unituxin), a monoclonal antibody that targets the ganglioside GD2, is the most effective immunotherapy for neuroblastoma. Dinutuximab is reported to improve the 2-year event-free survival of high-risk neuroblastoma patients from 46% to 66% [72]. Atezolizumab (Tecentriq) is an engineered monoclonal antibody designed against PD-L1. The safety, tolerability, pharmacokinetics, immunogenicity, and preliminary efficacy of this antibody is being evaluated by an early phase clinical trial (https://clinicaltrials.gov/ct2/show/study/NCT02541604). A recent report on a 6-year-old baby revealed that combination chemo-immunotherapy could be successfully used for high-risk NB relapsed after haploidentical stem cell transplantation (haplo-HSCT) [73].

In HLA class I positive neuroblastoma cell lines, PD-L1 is constitutively expressed, whereas PD-L2 is very rarely detected [69]. Furthermore, an induction in PD-L1 by IFN-γ was observed in neuroblastoma cell lines and in neuroblastoma engrafted nude mice. Importantly, PD-L1 was identified in metastatic neuroblasts isolated from bone marrow aspirates of high-risk neuroblastoma patients. PD-1 positive cells were mainly represented by $\alpha\beta$ T cells, as well as small populations of $\gamma\delta$ T cells and NK cells. The authors of this study concluded that PD-L1-mediated immune resistance mechanisms occur in metastatic neuroblasts and thus provide a biological rationale for blocking the PD-1/PD-Ls axis for immunotherapy.

PD-1 inhibitors have also been used in combination for neuroblastoma immunotherapy [69, 74–76]. For example, in combination with TLR ligands, the PD-L1 blockade was proposed as a promising immunotherapy for neuroblastoma [76]. Furthermore, pembrolizumab is more effective than docetaxel in PD-L1-expressing advanced NSCLC patients [77].

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However, only some subsets of patients benefit from PD-1/PD-L1 immune checkpoint blockade therapies. Therefore, it is essential to identify those populations that can benefit from PD-1/PD-L1 blockade therapy. Currently, PD-L1 expression in tumor specimens by IHC is the most commonly used biomarker for selecting patients with the possibility to respond to treatments [78]. However, conflicting results have been reported on PD-L1 expression. For example, over-expression of PD-L1 in melanoma is associated with poor clinical outcome [79]. Conversely, the PD-L1 over-expression in the context of CD8-positive cells is associated with a better prognosis [80]. Although these studies suggest the efficacy of PD-1 inhibitors against melanoma and NSCLC, the potential of these inhibitors against neuroblastoma has not been determined.

4. Inflammation and Neuroblastoma

While acute inflammation is beneficial, chronic inflammation leads to various chronic diseases including cancer. Various inflammatory molecules have been identified as a molecular mediator of cancer. These include pro-inflammatory transcription factors (NF-κB, STAT3), tumor necrosis factor (TNF), interleukin (IL)-1, IL-6, chemokines, cyclooxygenase (COX)-2, 5-lipooxygenase (5-LOX), matrix metalloproteinases (MMPs), vascular endothelial growth factor (VEGF), adhesion molecules, and numerous other molecules. Apart from inflammatory molecules, non-tumor cells such as tumor-associated macrophages (TAMs) are also known mediator of inflammation. The role of TAMs, macrophage migration inhibitory factor (MIF), chemokines and transcription factors in neuroblastoma pathogenesis are discussed in following sub-sections.

4.1. Tumor Associated Macrophages (TAMs)

Tumor-associated macrophages (TAMs) are cell types that are found near or within tumor masses. M1 and M2 are two major macrophage classes that are activated in response to dynamic stimuli. These macrophages are essential modulators of the immune response. The activation of M1 macrophages is observed in response to bacterial infection or interferon-γ (IFN-γ). This leads to enhanced antigen presentation and increased production of IL-12, IL-23, and reactive oxygen species (ROS) and anti-tumor effects. M2 macrophages are induced by interleukins (IL-4, -10, -13), and glucocorticoids, and promote growth and metastasis of neuroblastoma [81]. M2 macrophages can contribute to tissue remodeling, repair, and may protect tumor cells from apoptosis [82].

TAMs are fundamental to the progression of many tumor types including neuroblastoma [83–85]. Growing evidence suggests that TAMs can facilitate progression of neuroblastoma. Metastatic neuroblastoma patients are characterized by higher infiltration of TAMs. Neuroblastoma patients with an age of 18 months have higher expression of inflammationrelated CD16, CD33, IL6R, IL10, and FCGR3 than in patients diagnosed at the age of < 18 months [86]. MYCN non-amplified stage 4 patient's clinical reports have demonstrated that increased expression of TAM- associated genes such as CD14 and CD16 are significantly correlated with 5-year event-free survival [87]. Lymphocyte markers such as CD8A, CD4, and FOXP3 were not correlated with MYCN non-amplified stage 4 patient's clinical reports. Neuroblastoma cells cultured with primary monocytes are associated with

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rapid tumor growth in a xenograft mouse model. While administration of IL-6 neutralizing antibodies reduced tumor growth, over-expression of IL-6 was associated with increased tumor growth rates. The presence of TAMs was confirmed by immunohistochemistry. Furthermore, a significant increase in IL-6 was observed in tumor-infiltrated bone marrow [87]. IL-6 derived from bone marrow stromal cells (BMSC) is enhances progression of metastatic neuroblastoma [88]. When IL-6R-positive neuroblastoma cells are cultured in the presence of BMSC or recombinant human IL-6, an increase in proliferation is observed. Furthermore, IL-6R-positive neuroblastomas are protected from etoposide-induced apoptosis in the presence of BMSC or recombinant human IL-6. However, these responses were not observed in IL-6R-negative neuroblastoma cells [88]. A major limitation of the current neuroblastoma therapy is that over time neuroblastoma cells develop resistance to therapeutic agents [89]. Previous studies have demonstrated that TAMs can promote neuroblastoma growth, metastasis and the development of drug resistance [86]. Thus TAMs represent a negative prognostic factor for neuroblastoma [90–92]. However, the molecular mechanism by which TAMs contribute to tumorigenic effects is not properly understood.

4.2. Tumor-infiltrating lymphocytes (TILs)

Tumor-infiltrating lymphocytes (TILs) are group of mononuclear T cells infiltrated from tumor tissue found in most solid tumor including breast, colon, cervical, melanoma and neuroblastoma [93]. TILs are found in stroma within tumor area and contain T cells, B cells, NK cells and macrophages. TILs are important for diagnosis and prognosis in patients with many solid tumor including neuroblastoma [70, 94, 95]. A sub population of CD4+ cells in TILs shows detrimental effect on host *milieu* [96]. The histopathological specimen containing TIL could provide a decisive prognostic information in a wide variety of solid tumors especially neuroblastoma. TILs are assessed by standardized methodology on hematoxylin and eosin dye stained histological section based on the international immuno-oncology biomarker working group guidelines [97]. This standardized methodology may be used to evaluate clinical validity and utility of immunotherapy in neuroblastoma patients. Genetically modified TILs are preferred over conventional therapies in treating malignancies such as neuroblastoma [98].

4.3. Tumor Associated Mesenchymal Stem Cells (TMSCs)

Mesenchymal stem cells (MSCs) are multipotent stem-like cells that can differentiate into a variety of cell types [99, 100]. MSCs are known to produce a large range of cytokines, growth factors, proteins and can regulate survival, angiogenesis, immunomodulation, and drug resistance [101]. The primary and metastatic tumors are known to attract MSCs from bone marrow and other sites in their microenvironment. Conversely, in the bone marrow, MSCs attract tumor cells and contribute to a microenvironment that promotes tumor growth.

Cancer-associated fibroblasts (CAF) originating from MSC through the mediation of STAT3 and ERK1/2 pathways [102] are known to provide a favorable environment for neuroblastoma progression [103]. MSCs have also been used as a delivery vehicle for neuroblastoma therapy [104, 105]. For example, MSCs were used as carriers of oncolytic adenovirus and improved the efficacy of virotherapy for neuroblastoma by delivering the adenovirus to tumors and recruiting T cells [105].

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4.4. Macrophage Migration Inhibitory Factor (MIF)

MIF is an important pro-inflammatory mediator that links inflammation with cancer [106]. The expression of MIF in neuroblastoma is well documented [107]. MIF plays an essential role in both innate and acquired immunity. MIF is known to abrogate the functions of p53 leading to the promotion of tumor cell proliferation and angiogenesis, inhibition of apoptosis, and induction of cyclooxygenase-**2** (COX-2) through enhanced endothelial cell proliferation and differentiation [106, 108].

4.5. Chemokines and Transcription Factors

Chemokines are a family of secreted proteins that play an essential role in coordinating the inflammatory and immune response by specifically controlling leukocyte trafficking. Accumulating evidence suggests that chemokines can help with the migration, proliferation, and survival of tumor cells. Neuroblastoma cells are known to express C-X-C chemokine receptor type 4 (CXCR4) and the ligand, CXCL12 [109]. Furthermore, increased expression of CXCR4 correlates with advanced clinical stage and the presence of bone marrow metastases with poorer outcome [109]. The hypoxia-inducible transcription factors (HIF-1α, HIF-2α) are correlated with metastasis and development of drug resistance [110]. These transcription factors are differentially expressed in neuroblastoma cells [111]. While HIF-1α is transiently stabilized, prolonged expression of HIF- 2α is observed in neuroblastoma cells under hypoxic conditions [111].

NF-κB is a pro-inflammatory transcription factor that is constitutively expressed in most tumor types including neuroblastoma [112, 113]. It is comprised of five subunits including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel. Under normal conditions, NF-κB resides in the cytoplasm in association with its inhibitory subunit IκB. Upon stimulation, IκB subunit undergoes phosphorylation, ubiquitination, and degradation. This releases the p65-p50 subunit and allows it into the nucleus where it regulates the expression of NF-κB dependent target genes [114]. The phosphorylation of IκB can also lead to its dissociation from the trimeric complex without degradation. NF-κB can regulate the expression of over 500 cancer-related genes that are involved in various aspects of tumor development including transformation, survival, proliferation, invasion, angiogenesis, and metastasis [112, 115]. Therefore, NF-κB signaling pathway represents a potential target for therapeutic intervention. Agents that can inhibit protein tyrosine kinases, serine/threonine kinases, ubiquitination, proteasomes, acetylation, and DNA binding steps, have the ability to inhibit NF-κB activation. Although over 500 NF-κB inhibitors have been identified by preclinical studies, only bortezomib is used to treat multiple myeloma patients. Furthermore, the potential of bortezomib in neuroblastoma patients remains to be determined.

5. MiRNAs and Neuroblastoma

The role of miRNAs in regulating neuroblastoma pathogenesis is well established (Table 1). For example, miR-148a, miR-21, and miR-200a can modulate cell growth, migration, invasion, and apoptosis in neuroblastoma [116]. Similarly, miR-10b, miR-29a/b, miR-335, miR-7, and miR-338-3p are potentially associated with neuroblastoma progression [117]. miR-203 is known to inhibit the proliferation, migration, and invasion of neuroblastoma

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cells by targeting Sam 68 [118]. miR-337-3p and miR-584-5p can target matrix metalloproteinase (MMP) in neuroblastoma cells [37, 119]. miR-558 induces the transactivation of heparanase and promotes tumorigenesis in neuroblastoma [120]. The miR-17-92 polycistronic cluster is highly expressed in neuroblastoma tissues and serves as a marker for poor outcomes in patients [121]. miR-21, a well-known oncogenic miRNA, can promote proliferation and reduce chemo sensitivity in neuroblastoma cells [122]. miR-15a can also suppress the expression of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) a cysteine-rich protein with Kazal motifs and promoted the migration of neuroblastoma cells [123].

A study reported that 32 of 157 miRNAs in primary neuroblastomas are differentially expressed in favorable and unfavorable tumor subtypes [124]. miR-92a, miR-15b, miR-128a, and miR-628-3p were significantly increased in neuroblastoma cells. miRNA expression profile in low-risk and high-risk neuroblastoma patients has also been reported [125]. miR-92a, miR-15b, miR-128a and miR-628-3p are also known to be significantly altered and epigenetically silenced in metastatic neuroblastoma [126, 127]. miR-380-5p is over-expressed in neuroblastoma and is known to repress p53 and inhibit apoptosis in NB cells [128]. Furthermore, an over-expression in miR-380-5p is associated with poor outcomes in NBs [128]. Using the neuroblastoma cell line SH-SY5Y, miR-125b was found to play an important role in human neuronal differentiation [129]. Similarly, miR-10a and miR-10b are known to induce neuroblastoma cell differentiation by targeting the nuclear receptor corepressor 2 [130]. miR-340, a tumor suppressive miRNA is epigenetically silenced. Ectopic expression of this miRNA is known to induce differentiation and apoptosis in a context-dependent manner in neuroblastoma cells [131]. The inhibition of miR-18a in neuroblastoma cells led to the outgrowth of varicosity-containing neurites and the induction of sympathetic neuron differentiation markers [132]. MYCN knockdown could also induce neuroblastoma differentiation. During MYCN knockdown-mediated neuronal differentiation, 23 miRNAs were differentially expressed in neuroblastoma cells [133]. miR-21 was strongly up-regulated upon MYCN knockdown. However, miR-21 over-expression did not prevent the differentiation of neuroblastoma cells. Recently, we demonstrated that the exosomal miR-21 released from neuroblastoma cells could be transferred to human monocytes [116]. Similarly, miR-155 from human monocytes can be transferred to neuroblastoma cells. We previously shown that unique role of exosomal miR-21 and miR-155 in the cross-talk between neuroblastoma cells and human monocytes and in the development of resistance to chemotherapy[116]. Mechanistically, a novel exosomic miR-21/TLR8-NF-κB/exosomic miR-155/TERF1 signaling pathway was involved in the development of chemo resistance.

During the past decade, attempts have been made to develop miRNA-based therapeutics for neuroblastoma. One strategy is to block oncogenic miRNAs using oligonucleotides. In certain instances, nanoparticles have been used for the delivery of tumor suppressive miRNAs. For example, nanoparticles encapsulating miR-34a and conjugated to a GD2 antibody was found to facilitate tumor-specific delivery into mice [134]. Furthermore, a significant reduction in tumor growth, increased apoptosis and a reduction in vascularization were observed. miR-9 overexpression was found to inhibit invasion, metastasis, and angiogenesis of neuroblastoma cells *in vivo* [90]. Similarly, miR-145 can significantly

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inhibit the growth, invasion, metastasis, and angiogenesis of neuroblastoma cells by directly targeting HIF-2a [135]. miR-184 can significantly reduce tumor growth in an orthotopic murine model of neuroblastoma [136]. Similarly, an *in vivo* delivery of miR-380-5p antagonist can decrease tumor size in an orthotopic mouse model of neuroblastoma [128]. miRNAs can also be used in combination therapy for the clinical management of neuroblastoma. For example, miR-7-1 can enhance the inhibitory effects of 4-HPR [N-(4-hydroxyphenyl) retinamide] and EGCG (epigallocatechin-3-gallate) on neuroblastoma cells growth [137].

From above discussion, it is clear that miRNAs can be used as a target for neuroblastoma therapy. However, the potential of miRNAs in neuroblastoma patients remains to be explored. Extensive preclinical studies focused on safety and toxicity is necessary before a miRNA-based therapy can be considered for neuroblastoma patients.

6. LncRNAs and Neuroblastoma

Because of high specificity, dys-regulated expression pattern, and ease in the detection methods, lncRNAs can be used as a biomarker and therapeutic target for neuroblastoma. Some lncRNAs have been studied in the context of neuroblastoma (Table 2). N-Myc is a protooncogene encoded by the MYCN, which is a highly conserved and major oncogene in humans. N-Myc plays a crucial role during normal brain development [138]. However, MYCN amplification and N-Myc over-expression is associated with several cancer types, especially neuroblastoma [59–61]. The neuroblastoma patients with N-Myc amplification are prone to metastasis. N-Myc is also known to regulate the expression of lncRNAs such as T-UCRs and ncRNAs [139].

A study was aimed to examine lncRNA expression pattern by micro array in neuroblastoma cells after transfection with control or N-Myc-specific siRNA [140]. The linc00467 was identified as the novel lncRNA target of N-Myc. N-Myc suppressed linc00467 expression by direct binding to its promoter. Furthermore, gene silencing of linc00467 up-regulated the tumor suppressor gene DKK1, suppressed the viability and increased the apoptosis in neuroblastoma cells. These effects were reversed by the use of DKK1 siRNA. Thus, linc00467 can reduce neuroblastoma growth by modulation of DKK1 expression. Another study examined the expression pattern of lncRNAs and protein-coding genes between MYCN amplified and MYCN non-amplified NB patients [141]. A total of 6 lncRNAs were differentially expressed in neuroblastoma patients. MYCN amplification was found to up-regulate the expression of the lncRNA, SNHG1. SNHG1 was co-expressed with TAF1D (coding gene), and exhibited an association with poor patient survival. Furthermore, high expression of SNHG1 was predicted as an independent prognostic marker for event-free survival of patients. A robust expression of GAS5 is also reported in MYCN-amplified and non-amplified neuroblastoma cell lines [142]. The gene silencing of GAS5 produced defects in proliferation, apoptosis, and cell cycle arrest. The loss of GAS5 also induced p53, BRCA1, and GADD45A in neuroblastoma cell lines [142].

MYCN is also known to enhance lncRNAs expression by inducing epigenetic changes. For example, JMJD1A, a histone demethylase, has the potential of demethylating the lysine 9

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residue of histone H3 (H3K9) and thus can activate gene transcription. In one study, N-Myc was found to directly bind to the JMJD1A gene promoter and up-regulated the gene expression in N-Myc amplified human neuroblastoma cells [143]. Furthermore, JMJD1A up-regulated MALAT1 by inducing histone demethylation at its promoter. While JMJD1A and MALAT1 induced, use of DMOG (small molecule inhibitor of JMJD1A) was found to suppress the migration and invasion of neuroblastoma cells. Overall, these results suggest that N-Myc can modulate the neuroblastoma cell migration and invasion by modulating JMJD1A and MALAT1 expression.

Neuroblastoma cells like other cancer cells are characterized by hypoxic conditions. In one study, hypoxia was found to induce MALAT1 in neuroblastoma cell lines [144]. The gene silencing of MALAT1 was associated with a reduction in endothelial cell migration, invasion and vasculature formation, and down-regulation of the expression of fibroblast growth factor 2 (FGF2). Interestingly, an addition of recombinant FGF2 protein to the cell culture media reversed the effects of MALAT1 siRNA on vasculature formation. Overall, these data suggest that MALAT1 mediate its tumorigenic effects under hypoxic conditions by modulating the expression of FGF2 [144]. MALAT1 can also up-regulate Axl, which is a member of the receptor tyrosine kinase family and associated with neuroblastoma metastasis [145]. MALAT1 can also induce ERK/MAPK activation and neuronal differentiation in neuroblastoma cell lines [146].

The lncRNAs HCN3, linc01105, and MEG3 are known to regulate neuroblastoma pathogenesis. While a high expression of HCN3 and linc01105 was observed in neuroblastoma tissue, MEG3 expression was decreased [147]. The gene silencing of HCN3 and linc01105, and MEG3 over-expression was associated with an increase in apoptosis. Furthermore, linc01105 knockdown promoted cell proliferation, whereas MEG3 over-expression inhibited proliferation. These observations suggest that HCN3 and linc01105 act as oncogenes, while MEG3 is a tumor suppressor.

NBAT1 (neuroblastoma associated transcript 1) is a tumor suppressor lncRNA identified in neuroblastoma. It regulates cell proliferation and invasion by interacting with EZH2 (enhancer of zeste 2) [148]. Furthermore, NBAT1 can be used to predict clinical outcome of neuroblastoma patients [21, 148]. NBAT1 can also contribute to the aggressiveness of neuroblastoma by promoting proliferation and impairment in the differentiation of neuronal precursors [21].

The chromosome 6p22 has been reported as the most susceptible locus for the development of neuroblastoma [149]. This locus harbors several lncRNAs including the tumor suppressor, CASC15. The low-level expression of a short CASC15 isoform (CASC15-S) is highly associated with advanced neuroblastoma and poor patient survival [150]. Furthermore, attenuation of CASC15-S can increase extracellular matrix transcripts, cellular growth, adhesion and migration in neuroblastoma cells. The lncRNA, ncRAN is located on chromosome 17q25.1 and contains two splice variants: a long form (Nbla10727) and a short form (Nbla12061) [151]. An up-regulation in ncRAN has been reported in neuroblastoma patients that associate with poor prognosis [151]. The intergenic lncRNA loci are known to regulate the expression of adjacent protein-coding genes. Dali is an intergenic lncRNA that

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is transcribed downstream of the Pou3f3 transcription factor. The depletion of Dali can disrupt the differentiation of neuroblastoma cells [152]. Dali can also epigenetically regulate the expression of genes.

PVT-1 is a lncRNA that can regulate c-Myc over a long distance. One study examined the expression of PVT-1 and c-Myc in normal human tissues and transformed cells [153]. Although PVT-1 was restricted to a relatively low number of normal tissues, c-Myc mRNA was widely distributed. However, PVT-1 was highly expressed in many transformed cells including neuroblastoma that do not express c-Myc. Furthermore, PVT-1 promoter region contained two putative binding sites for Myc proteins.

Transcribed ultra conserved regions (T-UCRs), another class of novel lncRNA [154], exhibit absolute conservation in humans, rat, and mice [155]. T-UCRs are associated with neuroblastoma pathogenesis [156]. In one study, T-UCRs were found to possess prognostic significance [157]. This novel class of lncRNAs was associated with MYCN-amplification in neuroblastoma patients. Although seven T-UCRs (uc.279, uc.347, uc.350, uc.364, uc.379, uc.446, and uc.460) were up regulated in MYCN-amplified tumors, none of the T-UCRs were down regulated. Furthermore, T-UCRs were widely associated with cancer-related pathways such as proliferation, apoptosis, and differentiation. The significance of T-UCRs in neuroblastoma pathogenesis was demonstrated by another study [158]. Some other lncRNAs reported in neuroblastoma are Gomafu [159], H19 [160], CAI2 [161], lncUSMycN [162, 163], HOXD-AS1 [164], HOTAIR [165], and Paupar [166]. The lncUSMycN inhibit degradation of N-Myc mRNA as described in Figure 3.

Overall, it is clear that both up-regulation and down-regulation in lncRNAs expression pattern could be of diagnostic and prognostic significance for neuroblastoma patients. Additionally, lncRNAs can also be used as therapeutic targets. However, most conclusions are based on the modulation of gene expression that could also result from non-cancer conditions. In spite of several studies, none of the lncRNAs are recommended for use in neuroblastoma patients. Future studies should be focused more towards elucidating the clinical utility of these lncRNAs in neuroblastoma patients.

8. System biology and neuroblastoma

System biology is an interdisciplinary field to study the complex interaction within the biological system using computational and mathematical modeling. In 1952, British neurophysiologists Alan Lloyd Hodgkin and Andrew Fielding Huxley were created a mathematical model to explain the neuronal cell axon's action potential propagation [167]. This model described the interaction of cellular functions of sodium and potassium channels, and this was a landmark discovery to beginning computational system biology field [168]. It analyzed complex data sets such as genomics, proteomics, transcriptomics, metabolomics, glycomics, lipidomics from various experimental data sets to use computational tools. Cancer system biology deal with specific data sets such as patients samples, high-throughput patient's genome, cancer cell lines, xenograft models, next-generation sequencing, siRNA based screenings, somatic mutations and genome instability [169].

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The recent development of genome and proteasome level annotation of protein-protein interaction (PPI) networks facilitate functional cross-talk between genes in neuroblastoma [170]. Computational modeling was performed in neuroblastoma PPI network to elucidate the mutated genes effects on altered pathways in neuroblastoma [171]. The researchers have an opportunity to overlay patients and experimental data on to the network scaffold database such as BioGRID, STRING, and KEGG to analyze protein and gene interactions [172]. NeAT, an open source network analysis tool analyze and visualize the epigenetic, transcriptomic and metabolomics data may be an option to use neuroblastoma research [173]. Reverse-engineer networks approaches presume the interaction between data through Bayesian probabilistic models and pearson correlation coefficients[174]. This method has the capable the power to operate the human disease model like neuroblastoma and suitable for study the novel vital regulators play an essential role in neuroblastoma etiology and progression using large-scale multi-omics data [175].

It is challenging effect to apply system biology tools in human disease, but the ultimate goal was achieved through the collaborative efforts made with clinicians from all over the world. System biology promise of shedding new light to superior diagnosis to classify the virtual patient data and predict the outcome of suggested treatment on the basis of personalized neuroblastoma medicine. The system biology is more relevant to heterogeneous nature of neuroblastoma because it benefitted from the holistic and integrated approach to risk stratification. It provides realistic multi-scale *in silico* models of neuroblastoma and helps the clinical management and therapeutic design for the neuroblastoma patients.

9. Conclusions

The checkpoint molecules (PD-1, PD-L1), inflammatory molecules, lncRNAs, and miRNAs play a crucial role in neuroblastoma pathogenesis. These signaling molecules regulate various aspects of tumor development including transformation, survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells. Opdivo (nivolumab) and keytruda (pembrolizumab) are PD-1 inhibitors approved by FDA for advanced melanoma and non-small cell lung cancer. However, the efficacy of these inhibitors for neuroblastoma patients remains to be examined. Dinutuximab (unituxin), a monoclonal antibody that targets the ganglioside GD2, is the most effective immunotherapy for neuroblastoma. Atezolizumab (Tecentriq), an engineered monoclonal antibody against PD-L1, is currently being evaluated for its potential in neuroblastoma patients.

Although bortezomib, a potent NF-κB inhibitor is approved for multiple myeloma patients, its potential for neuroblastoma patients remains to be determined. An inhibition of oncogenic miRNAs/lncRNAs or delivery of tumor suppressive miRNAs/lncRNAs is a potential strategy for neuroblastoma therapy. However, delivery, stability and off-target effects are some of the limitations associated with miRNA/lncRNA-based therapeutics. Future studies should be focused towards improving the delivery options of miRNAs and lncRNAs for prolonged therapeutic efficiency and safety. It is also imperative to examine if the crosstalk between miRNAs/lncRNAs and PD-1/PD-L signaling pathways exist. The system biology approach will probably add significantly in this direction.

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In conclusion, immune-oncology has provided new hope to neuroblastoma patients. As discussed in this review, miRNA/lncRNA and immune checkpoint molecules could be used as biomarker and therapeutic target. The non-coding based biomarker has been developed for some cancer type. However, non-coding RNA based biomarker has yet to be approved for neuroblastoma. As for other cancer types, clinical trials in neuroblastoma are associated with high failure rates due to the expression of PD-L1 and other immune checkpoints in cancer cells as well as other cells of the tumor microenvironment. The use of reliable preclinical animal models will probably help to rapidly progress neuroblastoma field. New areas such as "Drug repurposing" should be explored to develop neuroblastoma therapy. We hope that the ongoing research across the scientific community will possibly help to place PD-1/PD-L1 and miRNA/lncRNA-based therapeutics as a potentially new facet for neuroblastoma therapy.

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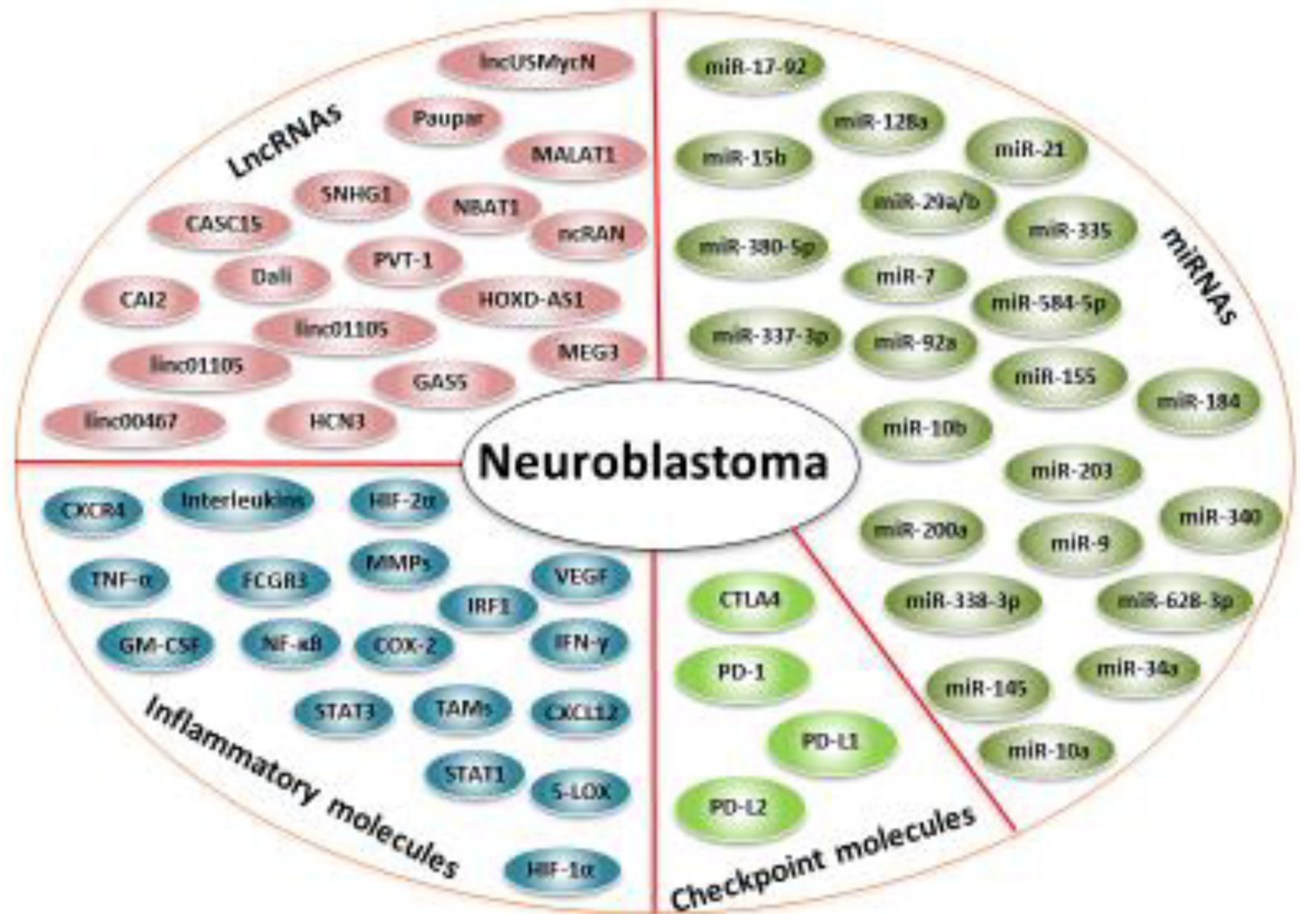

Figure 1. Potential Biomarkers and therapeutic targets of Neuroblastoma

Abbreviations: 5-LOX: 5-lipoxygenase, CAI2: CDKN2A/ARF intron 2, CASC15: cancer susceptibility candidate15, COX-2: cyclooxygenase-2, CTLA4: cytotoxic T-lymphocyte associated protein 4, CXCL12: C-X-C motif chemokine 12, CXCR4: C-X-C chemokine receptor 4, FCGR3: Fc fragment of IgG receptor III, GAS5: growth arrest special 5, HCN3: hyperpolarization-activated cation nucleotide-gated isoform 3, HIF-1α: hypoxia-inducible factor 1-alpha, HOXD-AS1: HOXD cluster antisense RNA 1, IRF1: interferon regulatory factor 1, linc00467: long Intergenic non-protein coding RNA 467, linc01105: long intergenic non-protein coding RNA 1105, lncUSMycN: lncRNA upstream of *MYCN*, MALAT1: metastasis associated lung adenocarcinoma transcript 1, MEG3: maternally expressed 3, miR: microRNA, NBAT1: neuroblastoma associated transcript 1, ncRAN: non-coding RNA expressed in aggressive neuroblastoma, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, PD-1: programmed death-1, PD-L1: programmed death-ligand 1, PD-L2: programmed death-ligand 2, PVT-1: plasmacytoma variant translocation 1, SNHG1: small nucleolar RNA host gene 1, STAT-3: signal transducer and activator of transcription 3, TAMs: tumor-associated macrophages, TNF-α: tumor necrosis factor-alpha, VEGF: vascular endothelial growth factor.

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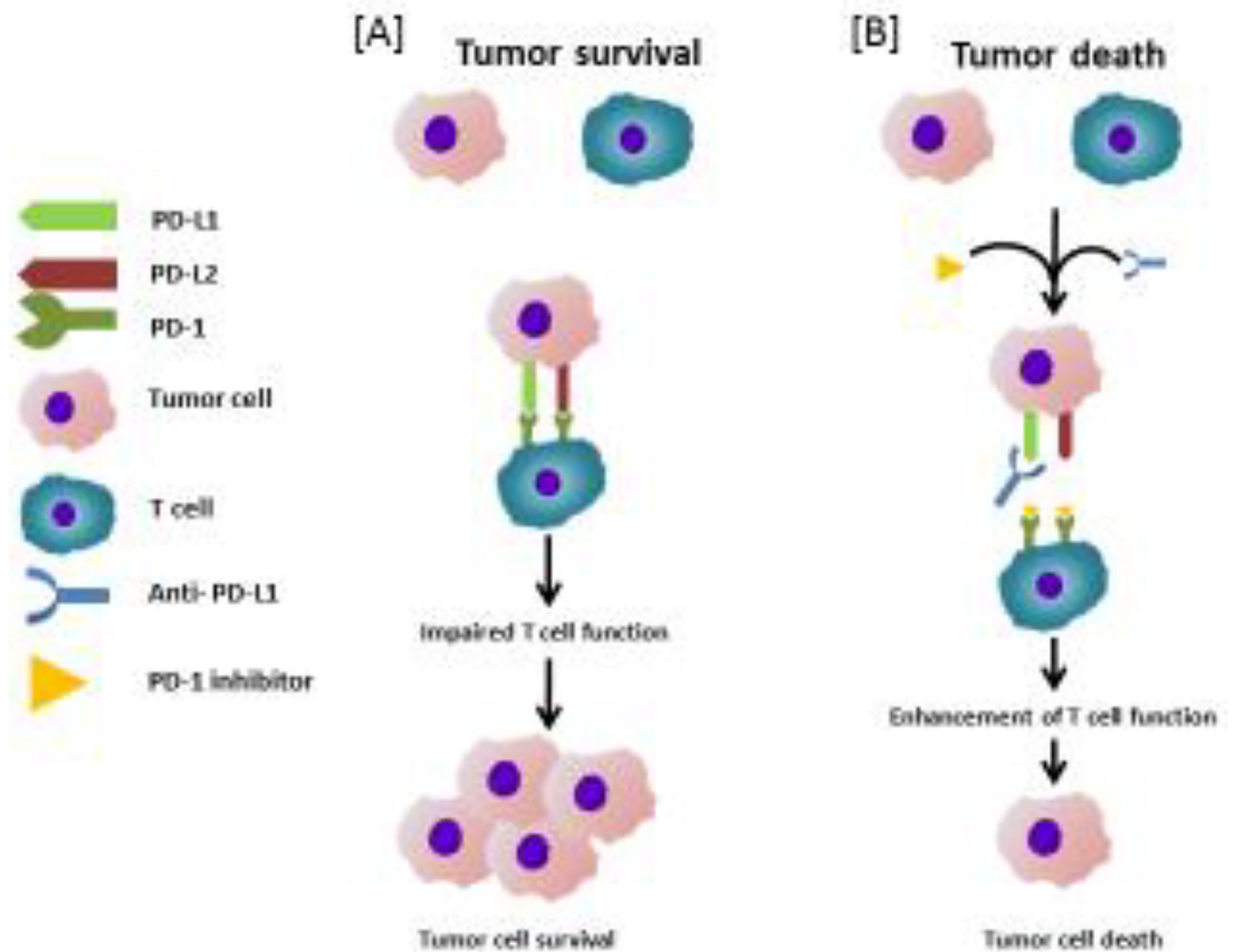

Figure 2. The molecular basis for the action of PD-1and PD-L1 based therapy

[A] PD-1 is expressed by T-cells, while PD-L1/PD-L2 is expressed by tumors. In neuroblastoma, interaction of PD-1 with PD-L1/PD-L2 suppresses T-cells function. [B] The PDL-1 antibodies act by blocking the interaction of PD-L1 with PD-1without affecting PD-L2/PD-1 interaction. This enhances the T cell function leading to anti-tumor activity.

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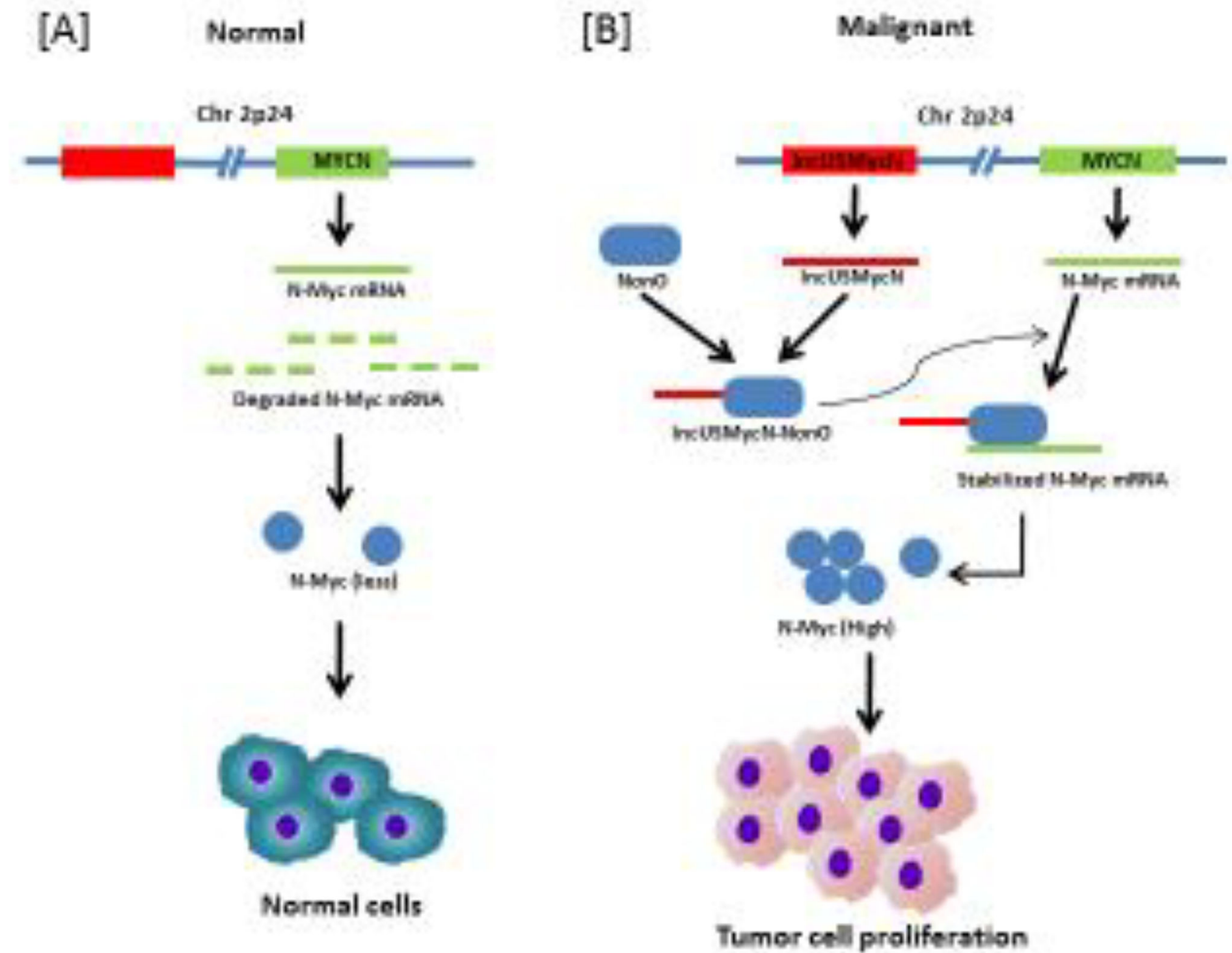

Figure 3. The lncUSMycN act by inhibiting degradation of N-Myc mRNA

[A] In normal, cell, N-Myc undergoes degradation at post transcriptional and post translation level. [B] In neuroblastoma cells, MYCN oncogene harbor lncUSMycn, a lncRNA at 14 kb upstream of the gene. The lncUSMycn act as a scaffold for the RNA binding protein NonO. This facilitate the interaction between NonO and MYCN. This results in the stabilization of MYCN transcript and eventually proliferation of tumor cells

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Table 1

A list of miRNAs dys-regulated in neuroblastoma and their functions

Column1	Column2	Column3	Column4
miRNA	Expression	Function/Clinical implication	Reference
miR-9	↓	Inhibited invasion, metastasis, and angiogenesis of NB cells by targeting MMP-14	[90]
miR-10a		Induced neural differentiation by suppressing NCOR2 and MYCN in neuroblastoma cells	[130]
miR-10b		Induced neural differentiation by suppressing NCOR2 and MYCN in neuroblastoma cells	[130]
miR-15a		Induced NB migration by targeting RECK and regulating MMP-9	[123]
miR-17-92		Inhibited TGF-β signaling; induced proliferation and adhesion of NB cells	[176]
miR-34a	↓	Inhibited NB growth when administered to the mice in conjugation with GD2 antibody	[134]
miR-145	↓	Inhibited the growth, invasion, metastasis and angiogenesis of NB cells by targeting HIF-2α	[135]
miR-155		Contributed to the development of chemoresistance by neuroblastoma cells	[116]
miR-184	↓	Inhibited tumor growth in an orthotopic NB murine model	[136]
miR-203	↓	Inhibited proliferation, migration and invasion of NB cells by targeting Sam 68	[118]
miR-335	↓	Suppressed invasion of NB cells by targeting TGF-β signalling pathway	[177]
miR-337- 3p	↓	Suppressed nuroblastoma progression by repressing MMP-14	[37]
miR-340	↓	Induce differentiation and apoptosis in a context dependent manner in neuroblastoma cells	[131]
miR-380-5p		Repressed p53 and inhibited apoptosis in NB cells; associated with poor outcome in NBs	[128]
miR-584-5b	↓	Exerted tumor suppressive functions in NB cells by suppressing MMP-14	[119]

Abbreviations: : up-regulation, ↓: down-regulation, HIF-2α: hypoxia-inducible factor 2 alpha, MMP: matrix metalloproteinase, MYCN: v-myc myelocytomatosis viral related oncogene, NB: neuroblastoma, NCOR2: nuclear receptor co-repressor 2, RECK: reversion-inducing cysteine-rich protein with kazal motifs, Sam 68: src-associated in mitosis 68, TGF-β: transforming growth factor beta

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Table 2

A list of lncRNAs dys-regulated in neuroblastoma and their functions

Column1	Column2	Column3	Column4
lncRNA	**Expression**	**Function/Clinical implication**	**Reference**
linc00467	-	Reduced neuroblastoma growth through modulation of DKK1	[140]
SNHG1		Associated with event-free survival of patients	[141]
GAS5		Regulated expression of p53, BRCA1, and GADD45A in neuroblastoma cells	[142]
MALAT1		Induced migration and invasion of neuroblastoma cells	[143]
MALAT1		Modulated cell migration, invasion and vasculature formation by down-regulating FGF2 in	[144]
		neuroblastoma cells under hypoxic conditions	
MALAT1		Up-regulated Axl and induced invasion and migration of neuroblastoma cells	[145]
MALAT1		Induced ERK/MAPK activation and neuronal differentiation in neuroblastoma cell lines	[146]
HCN3		Regulated apoptosis and proliferation in neuroblastoma cells	[147]
linc01105		Regulated apoptosis and proliferation in neuroblastoma cells	[147]
MEG3	↓	Regulated apoptosis and proliferation in neuroblastoma cells	[147]
NBAT1	↓	Regulated proliferation and invasion of neuroblastoma cells by interacting with EZH2;	[148]
		associated with poor clinical outcome in patients	
NBAT1	↓	Contributed to the aggressiveness of neuroblastoma by promoting proliferation and	[21]
		an impairment of differentiation of neuronal precursors	
CASC15	↓	Regulated extracellular matrix transcripts, adhesion, growth, and migration of neuroblastoma cells	[150]
ncRAN		Associated with poor prognosis of neuroblastoma patients	[151]
Dali	-	Regulated the differentiation of neuroblastoma cells	[152]
PVT-1		Regulated expression of Myc proteins	[153]
CAI2		Associated with high-risk and clinical outcome of neuroblastoma patients	[161]
lncUSMycN	-	Regulated N-Myc expression and neuroblastoma oncogenesis in mice model	[162]
lncUSMycN		Induced NCYM expression; correlated with poor prognosis of neuroblastoma patients	[163]
HOXD-AS1	-	Regulated RA-induced differentiation and oncogenesis in mice model expression of genes associated	[164]
		with angiogenesis and inflammation in SH-SY5Y neuroblastoma cells	
Paupar	-	Induced differentiation of neuroblastoma cells	[166]

Abbreviations: -: up-regulation, ¯: down-regulation, Axl: AXL receptor tyrosine kinase, BRCA1: breast cancer 1, DKK1: dickkopf WNT signaling pathway inhibitor 1, ERK: extracellular signal-regulated kinase, MAPK: mitogen-activated protein kinase, EZH2: enhancer of zeste homolog 2, FGF2: fibroblast growth factor 2, GADD45A: growth arrest and DNA damage-inducible protein 45 alpha, Myc: myelocytomatosis oncogene, NBAT1: neuroblastoma associated Transcript 1, RA, retinoid acid

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Table 3

A list of immunotherapy based clinical trials for neuroblastoma

Columnl	Column2	Column3	Column4
Trials identifier No.	**Trial status**	**Drug/agent used**	**NB patient feature**
NCT02169609	Active, not recruiting	Dinutuximab (Ch 14.18) GM-CSF and IL-2	high-risk neuroblastoma
NCT02573896	Yet to recruit	ch14.18/Lenalidomide	relapsed refractory neuroblastoma
NCT01183897	Active, not recruiting	Hu3F8/GM-CSF and 13-Cis-Retinoic Acid	primary refractory neuroblastoma in bone marrow
NCT03033303	Recruiting	Hu3F8/GM-CSF Isotretinoin	high-risk neuroblastoma with first remission
NCT01183884	Active, not recruiting	3F8/GM-CSF 13-Cis-Retinoic Acid	high-risk neuroblastoma with second or greater remission
NCT01183429	Active, not recruiting	3F8/GM-CSF 13-Cis-Retinoic Acid	non-myeloablative therapy with high-risk neuroblastoma in first remission
NCT02765243	Recruiting	4SCAR-GD2 T cells	refractory or recurrent neuroblastoma
NCT02311621	Recruiting	genetically modified T cells to express CAR	recurrent or refractory neuroblastoma
NCT01183416	Active, not recruiting	High-dose 3F8/GM-CSF and 13-cis-retinoic acid	autologous stem-cell transplantation after myeloablative therapy first remission
NCT03242603	Recruiting	Anti-GD2/ NK Cells	high-risk neuroblastoma
NCT02130869	Recruiting	CD133+ selected autologous stem cell infusion/hu 14.18K322A	high-risk neuroblastoma
NCT02919046	Recruiting	GD2-targeted CAR-T cells	Relapsed or refractory neuroblastoma
NCT02173093	Recruiting	GD2 bispecific antibody	Children with neuroblastoma and osteosarcoma

Abbreviations: GM-CSF: granulocyte-macrophage colony stimulating factor, NK: natural killer cells, GD2: disialoganglioside, Hu3F8: humanized 3F8 monoclonal antibody; CAR: chimeric antigen receptor