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Close Neighbours on Chromosome 2 the ALK and MYCN Genes. Implications for Targeted Therapeutics in Neuroblastoma

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1. Introduction

Neuroblastoma is a neural crest-derived embryonal malignancy of the postganglionic sympathetic division of the peripheral autonomic nervous system. It is the most frequent extra cranial solid malignancy of childhood and the most common cancer in children less than one year of age. It accounts for 7% of childhood cancers and 15% of all childhood cancer deaths. It most commonly occurs sporadically but familial cases can also occur with one subdivision attributable to germline mutation of the PHOX2B gene and another due to germline ALK gene mutations [1,2,3,4]. Recurring chromosomal aberrations are detected in this disease and three major genomic subdivisions represent greater than 80% of cases; i.e. hyperdiploid neuroblastoma; near diploid neuroblastoma that has 17q gain and 11q deletions; and MYCN amplified neuroblastoma with 17q gain and 1p deletion [5,6,7,8]. A whole-genome analysis and assessment of structural defects in 87 primary neuroblastomas found few recurrent amino-acid changing mutations, with ALK mutations in 6%, but identified local shredding of chromosomes (chromothripsis) in 18% of high stage neuroblastomas. High stage tumours that did not have amplifications of MYCN had recurrent structural alterations in genes involved in cone stabilisation and neuritogenesis [9]. Indeed in this study tumours with a genomic signature involving defective genes responsible for neuritogenesis or growth cone guidance were mostly aggressive high-stage tumours.

Activating mutations of the anaplastic lymphoma kinase (ALK) genes and amplification of the MYCN oncogene can occur in neuroblastoma. The ALK gene resides on chromosome 2p23 centromeric to the MYCN locus on chromosome 2q24. Approximately 2-3% of cases of neuroblastomas have amplification of ALK and these occur almost invariably concomitant

with amplification of MYCN [10]. However, amplification of the ALK gene without concomitant MYCN gene amplification can occur, for example a particularly informative case of a neuroblastoma with a high level amplicon involving and solely limited to the ALK gene was described by French investigators in 2008 in one of the landmark papers that established the importance of ALK in neuroblastoma [11]. Activating ALK mutations are found in 80% of familial cases and 6-11% of sporadic cases. It is considered that once the expression of wild type ALK exceeds a certain threshold it can have oncogenic activity[10]. Twenty percent of all cases of neuroblastomas have amplification of MYCN which is associated with a worse prognosis and can be used to stratify treatment. This chapter appraises the current knowledge and potential therapeutic implications arising from ALK and MYCN abnormalities in neuroblastoma. In a concluding reflection the chromosomal proximity and interaction of these genes as well as the potential for therapeutic advancement in neuroblastoma is discussed.

2. The anaplastic lymphoma kinase gene in neuroblastoma

2.1. The ALK gene

The anaplastic lymphoma kinase gene is a 200kDa member of the insulin receptor super family. It is an orphan tyrosine kinase receptor and has homology with the MET oncogene and the neurotrophin receptor. It is normally expressed by the developing nervous system and at a much lower level in the nervous system of adults [12]. In mouse embryo studies *Alk* transcripts were detected in the central nervous system (CNS) and peripheral nervous system. E15 embryos had expression in the Gasserian ganglion of the trigeminal nerve (cell bodies of V₁, V₂ and sensory component V₃) as well as the superior cervical ganglion, posterior root ganglia of the spinal cord and the myenteric plexus of the enteric nervous system. In the 1 week old mouse *Alk* transcripts are clustered in particular neuronal regions in the CNS such as the mesencephalon, thalamic nuclei which act as relay stations for nerve impulses and olfactory bulb, mitral cells and tufted cells that receive primary afferents from olfactory epithelial neurones. Relatively high levels of *Alk* transcripts were present in the superior colliculus, which is the centre of visual sensation and the red nucleus, a crucial part of the rubrospinal tract which regulates the contraction of flexor muscles [13]. Lower levels were detected in the hypothalamus, inferior colliculus, subiculum, cerebral cortex and cerebellum (Purkinje's cells and Golgi cells). Interestingly one patient in a phase 1 study of an ALK inhibitor in children with relapsed/refractory ALK-driven tumors (57 evaluated for toxicity) developed grade 3 dizziness [14]. It remains speculative as to whether it is possible to attribute it to cerebellar Alk expression based on the finding of low level *Alk* expression in the murine cerebellum.

The anaplastic lymphoma kinase gene has a restricted pattern of expression in adults in that it is expressed in testis, small intestine and brain but is not expressed in normal lymphoid tissue. It has been shown to be important in the pathogenesis of ALK positive anaplastic large cell lymphoma, inflammatory myofibroblastic tumours and adenocarcinoma of the lung (NSCLC) [15]. Its importance in cancer was first described in 1994 in anaplastic large cell lymphoma where a translocation (2; 5) (p23; q35) fuses NPM a non-ribosomal nucleolar

phosphoprotein with ALK in 50-75% of cases [16]. The most frequent abnormality involving ALK in non-small cell lung cancer is an inversion of a segment of chromosome 2 creating a chimeric fusion gene involving ALK and the Echinoderm microtubule-associated protein; ALK-EML4 [17]. In inflammatory myofibroblastic tumours, tropomyosin TMP3 or TMP4 create fusion oncoproteins with ALK. Clonal rearrangements at chromosome 2p23 occur in 50% of these tumours [18]. More recently ALK protein expression has been seen in rhabdomyosarcomas with 81% of cases of the alveolar subtype having strong cytoplasmic staining for ALK compared to 31% of cases of the embryonal subtype. These subtypes have gene copy number gain of 88% and 52% respectively. In the embryonal subgroup ALK aberrations were associated with disease progression and outcome [19].

The ALK receptor is a dependence receptor and in the absence of ligand enhances apoptosis by autologous cleavage by caspase [12]. Therefore ALK belongs to the subcategory of oncogenes in which inappropriate expression in a cellular context or in the absence of ligand can induce apoptosis. This is considered to be a mechanism to abrogate frequent tumour formation if oncogenes become deregulated. Myc is also considered to be an established comparator gene in this respect. Additionally in the visual system of *Drosophila* ALK in the presence of a ligand appears essential for axonal guidance. It is known that perturbations of the visual system, in particular in the first 5 years of life can cause amblyopia [20, 21]. Also, in an expanded cohort study of 82 adults with ALK positive NSCLC treated with crizotinib, mild visual disturbances were reported by 41% of patients. These were most frequently described as trails of light following moving objects particularly seen with changes in ambient lighting usually improving with duration of time receiving treatment [22]. It may be the case that the aforementioned finding of *Alk* transcripts in the superior colliculus of the 1 week old mouse is relevant as the superior colliculus is important for saccadic and smooth pursuit eye movements. Though not described to date in patients with neuroblastoma treated with ALK inhibitors, given the age demographics of patients with neuroblastoma it is the author's contention that clinicians conducting ongoing trials need to be cognisant of the theoretical potential to damage the developing neuroanatomical visual system with resulting amblyopia. The visual system includes the optic nerves, optic chiasma and optic tracts; the lateral geniculate body a swelling beneath the posterior projection of the pulvinar of the thalamus and the geniculoclarine tract which originates within the lateral geniculate body and terminates at the calcarine sulcus of the medial surface of the cerebral hemisphere. Evaluation of the regional ALK status of this system is a subject for further research.

2.2. ALK in neuroblastoma

Neuroblastoma is considered to be a malignancy derived from the embryonic neural crest. Evidence of neuroblastoma being derived from neural crest progenitor cells are (i) neuroblastoma primary sites are anatomically consistent with arising from the sympatho-adrenergic lineage of neural crest differentiation (ii) The gene expression patterns of neuroblastomas and neural crest progenitor cells are similar [23] (iii) In situ neuroblastomas occur in the adrenal glands of 1/200 newborns with most spontaneously regressing later in life and there is a histological similarity to residual nests of sympathogonia [24].

A small proportion of cases of neuroblastoma display autosomal dominant Mendelian inheritance patterns and a familial history of neuroblastoma is elicited in 1-2% of cases [25, 26, 27]. There is a standardized incidence ratio of 9.7 for disease occurrence in siblings of index cases [28]. Previously it had been recognized that neuroblastoma can arise in the context of concurrent neurocristopathies related to abnormal development of neural-crest derived tissues such as concomitant Hirschsprung's disease or central congenital hypoventilation syndrome. Nonsense and missense mutations of the homeobox gene PHOX2B were shown to predispose to this abnormality of the sympathoadrenal lineage [4, 29, 30].

In October 2008 four articles were published in the journal *Nature* that established the importance of aberrations in ALK as a feature of some neuroblastomas [11,31,32,33]. Mossé and colleagues of Children's Hospital Philadelphia found that ALK is a major neuroblastoma predisposition gene with germline ALK mutations accounting for most cases of familial neuroblastoma [31]. Using a whole-genome scan of neuroblastoma pedigrees (8 unrelated families) a significant linkage signal at 2p23-24 was identified. Re-sequencing 194 high risk neuroblastomas found somatically acquired ALK mutations in 12.4% of cases. Most mutations mapped to the kinase domain of ALK and caused constitutive phosphorylation. Cell line studies also showed that knockdown of ALK mRNA caused growth inhibition suggesting that therapeutics targeting ALK may have clinical efficacy in neuroblastoma.

In another study lead by investigators at Dana Farber Cancer Institute, Boston mutations of ALK were found in 8% of primary neuroblastomas [32]. A genome wide study of primary neuroblastomas identified amplification of the ALK gene. Analysis of 94 tumours with amplification of MYCN found 14 (15%) with concomitant ALK amplification. This was not identified in 51 tumors without MYCN amplification. DNA resequencing of the ALK open reading frame identified in primary neuroblastomas found 5 non-synonymous sequence variations in the ALK tyrosine kinase domain in 7 of 93 samples (8%). The most frequent mutation which was in 4.3% (4) of the 93 cases was a cytosine to adenine alteration in exon 23 causing a phenylalanine to leucine substitution in codon 1174 (F1174L). This mutation was also found in 3 different neuroblastoma cell lines. Three of the mutations were somatic and 2 were germline. Interestingly 4 of the 5 identified mutations involved residues which correspond to those that are affected by activating EGFR gene mutation. The ALK 1174 residue correlated with V769 in EGFR and ERBB2 [34]. A F1245C ALK mutation correlated with the L833V mutation in EGFR, which is a gefitinib resistant mutation in NSCLC. The R1275Q mutation is positioned adjacent to the homologous position of L858R in EGFR, the most common mutation of EGFR in NSCLC [35, 36]. ALK cDNA encoding either the F1174L or the R1275Q variants transformed interleukin-3 dependent murine hematopoietic Ba/F3 cell lines to cytokine independent cell growth. Furthermore these autonomously growing cells were sensitive to the ALK inhibitor TAE684. Additionally 2 human neuroblastoma cell lines were sensitive to TAE684. It has been observed by Mossé and colleagues that there are disorder involving oncogenes such as RET in medullary thyroid cancer arising in the context of multiple endocrine neoplasia type 2 and MET in papillary carcinoma of the kidney that are analogous to the sequential 'two hit model' of Knudson. Knudson's was first used to describe retinoblastoma arising from aberrations of a tumor suppressor gene. In the oncogene 'two hit' model the

second hit is a somatically acquired duplicate of the mutant allele or mutant gene amplification [37]. In neuroblastoma oncogenic activation of ALK can occur by mutation of the tyrosine kinase domain and for example with respect to a 'second hit' in the Dana Farber led study, ALK gene amplification was found in 15% of 94 tumours with MYCN amplification.

French investigators assessed ALK copy number variation by comparative genomic hybridization in 592 cases of neuroblastomas [11]. Within this group 26 cases (4.4%) had in excess of a twofold copy number increase of ALK and a further 135 cases (22.8%) had lower level gains. The level of expression of ALK is strongly correlated with copy number [38]. Within the genomic analysis a subcategory without MYCN amplification was assessed using 100K single nucleotide polymorphism arrays. A notable case within this cohort was an instance of neuroblastoma with a high level amplicon involving and solely limited to the ALK gene. Also a series of 28 cell lines and 115 tumor samples found 16 ALK mutations grouped into 2 main hotspots. The F1174L mutation was seen in one primary tumor but was more frequent in cell lines suggesting that this particular mutation confers an *in vitro* growth advantage. Cell lines are usually over-represented by metastatic tumors with a greater propagating potential and this finding may be vicarious evidence of the F1174L mutation being correlated with a poorer clinical outcome. Finally a Japanese study identified 8 novel missense mutations in the ALK gene in 13 out of 215 cases of neuroblastoma (6.1%) and 8 of 33 (33%) neuroblastoma cell lines. The mutated kinases could transform NIH3T3 fibroblasts and form tumors in nude mice [33].

These four studies were a landmark advance in the field, however later a European group of international collaborators performed a meta-analysis of 709 neuroblastomas (254 new cases and 455 previously published) to comprehensively described the correlation of ALK mutation type and frequency with clinical and genomic factors. They also assessed the prognostic significance of ALK copy number and expression [39]. Mutations in ALK were detected in 6.9% of cases with a mutation frequency of ALK of 5.7% in favorable (International neuroblastoma Staging System INSS 1, 2 and 4s) cases and 7.5% in unfavorable tumors (INSS 2 and 4). There was no statistical difference with respect to mutation frequency between the favorable and unfavorable groups ($P=0.087$). Mutation hot spots R1275Q (49%) and F1174L (34.7%) were observed within the mutated cases. However the F1174L mutations occurred in a greater proportion of the MYCN amplified cases ($P=0.0001$) and the concurrence of a F1174L mutation in a MYCN amplified neuroblastoma was found to confer an especially poor prognosis. It was described that there was a skewed ALK mutation spectrum within the MYCN amplified cohort with over-representation of the F1174L mutation. F1174 mutated ALK was present in 1.3% of single copy MYCN tumors compared with 6.1% of MYCN amplified tumors. To consider it another way, within the 17 cases of F1174 ALK mutated neuroblastoma 58.8% had amplification of MYCN compared to a rate of 21.6% in cases of neuroblastoma with wild type ALK. There also was no difference in the frequency for MYCN amplification between the R1275Q cases and wild type ALK. The skewed distribution of F1174L mutations being overrepresented in MYCN amplified cases of neuroblastoma was also confirmed in 27 neuroblastoma cell lines most of which were MYCN amplified. Five had the F1174L mutation with only one case of R1275Q mutant neuroblastoma found. F1174 mutated neuroblastoma compared to the R1275Q

mutant variant had a greater transforming ability with a higher amount of auto phosphorylation. Gain in Chr. 2p that included ALK (91.8%) also was correlated with poor survival.

2.3. Possible new treatments for neuroblastoma that target ALK

Crizotinib is an orally bio-available small molecule that inhibits the tyrosine kinase activity of ALK and c-Met which is approved by the U.S. Food and Drug administration for the treatment of cases of NSCLC that have rearrangements of the ALK gene. Crizotinib competes with adenosine triphosphate to bind to the ALK tyrosine kinase. The two most common ALK mutations in neuroblastoma are F1174L and R1275Q and both mutations promote autophosphorylation of the ALK tyrosine kinase region. F1174 mutated ALK in particular is a more lethal variant. Using neuroblastoma cell lines and xenograft models it has been shown that different ALK mutations can affect both kinase activity and inhibitor sensitivity [40]. Both F1174L and R1275Q ALK mutations cause amino acid substitutions in the intracellular tyrosine kinase domain of the ALK receptor and constitutively activate the ALK tyrosine kinase domain. Neuroblastoma cell lines and xenograft mouse models that expressed R1275Q-ALK are sensitive to crizotinib. By comparison F1174L mutated ALK cell lines were relatively resistant to crizotinib. The $K_{m,ATP}$ for F1174L of $\sim 0.127Mm$ was approximately 2.3 times lower than the $K_{m,ATP}$ of 0.326 for the R1275Q mutant ALK variant. The F1174L mutation combines the characteristics of an activating gene mutation and a resistance mutation, increasing k_{cat} and maintaining a wild type like $K_{m,ATP}$. The derived overall inference of these findings is that comparative crizotinib resistance of the F1174L mutant ALK is due to greater ATP binding affinity and it is hoped that the resistance may be overcome by increasing the doses of crizotinib or using ALK inhibitors with increased affinity to the ALK tyrosine kinase domain.

The U.S. National Cancer Institute is sponsoring a phase I/II study of crizotinib in children with relapsed or refractory solid tumors and anaplastic large cell lymphoma. The phase I study was presented at the American Society of Clinical Oncology Annual Meeting in Chicago, June 2012 (Protocol IDs: COG-ADVL0912, ADVL0912, NCT00939770) by Yael Mossé; abstract 9500 [14]. The study enrolled 70 patients with confirmed ALK fusion proteins, mutations or amplification with 57 fully evaluable for toxicity. This was a heterogeneous study population with respect to cancer type and included other cancers in addition to neuroblastoma. Six different dose levels of crizotinib were evaluated using the rolling-six design and dosing was bid on 28 day cycles without interruption. The recommended phase II dose that emerged was 280mg/m²/dose. Seven of eight (88%) of patients with anaplastic large cell lymphoma had a complete response (2 differing dose levels). 27 patients in the trial had neuroblastomas. Within the cohort of neuroblastoma cases with a known ALK mutation (n=8), one patient had a complete response and 2 had stable disease. Of the 19 patients with neuroblastoma and an unknown ALK status, 1 had a complete response and 6 had prolonged stable disease.

With respect to toxicity there were two grade 5 cases of haemorrhage within the central nervous system in patients with neuroblastoma and the protocol was updated to exclude patients with a previous history of central nervous system involvement. Other toxicities observed within the study and not necessarily within the neuroblastoma subgroup were grade 4 transaminitis (n=1), grade 4 neutropenia (n=1) and grade 3 dizziness (n=1). A discussant at the meeting

Thomas Gross of The Ohio State University Nationwide Children's Hospital referenced some Crizotinib toxicities observed in adults with NSCLC including gastrointestinal complaints, transient vision disorders, self-limited lower testosterone levels and rare renal cysts. He felt that these toxicities necessitated further investigation in the paediatric population. Regarding efficacy he noted that there can be variability in oncogenic partners with ALK within chimeric fusion genes in differing disease types that may partly account for the rates of complete responses seen in different malignancies. He also observed that some responses were seen in ALK negative cases in the phase 1 trial. Phase II data on the efficacy and toxicity of Crizotinib in neuroblastoma will be required.

3. The MYCN gene in neuroblastoma

3.1. The MYCN family

MYC is a pleiotropic evolutionary conserved family of basic helix-loop-helix leucine zipper transcription factors, comprising c-Myc, L-Myc and N-Myc [41, 42]. These transcription factors regulate the expression of ~15% of all genes [43, 44]. MYC proteins have numerous roles in malignancy with roles of special importance in neuroblastoma being that of driving cellular proliferation and angiogenesis while concurrently inhibiting apoptosis and maintaining the neoplastic 'stem cell' compartment. MYCN is amplified in ~20% of cases of neuroblastoma and amplification of MYCN is independently correlated with a higher disease stage and a poor clinical outcome. It is amplified in 40% of cases with a high risk phenotype. It has been contended that targeting Myc is a therapeutically attractive strategy to treat cancer but difficulties persist. These include (i) Myc exerts effects by protein-protein and protein-DNA interactions. Small molecule inhibitors have not usually been effective in this context. (ii) Most Myc aberrations in malignancies are not due to intrinsic abnormalities of Myc but rather due to 'upstream' aberrant oncogenic signals causing its induction (iii) Myc is required for stem cell compartment maintenance and proliferation in normal tissues with a theoretical concern of serious bystander tissue toxicities [45] (iv) Analysis of the role of c-Myc and N-Myc in cancer is difficult to assess in tissues as embryonic lethality is conferred by germline deletion of these genes. Much of the data on the consequences of inhibiting Myc is derived from conditional knockouts of the c-Myc gene using Cre mediated recombination which can be very variable and unpredictable in the extent of c-myc deletion in the cells targeted (iv) Conditional knockout models are irreversible and therefore not good representative models of transient inhibition of Myc conferred by inhibitor medications.

One particularly important experimental example of Myc inhibition was in an adenocarcinoma of lung murine model [45]. Omomyc is a competitive inhibitor of Myc-dependent gene transcription by preventing the binding of Myc to its consensus E-box CACGTG DNA elements thereby preventing Myc binding to its obligate dimerization partner Max. This prevents the transactivation of its target genes. Omomyc may also augment Myc-dependent trans repression [46, 47]. The LSL-Kras^{G12D} murine model of NSCLC has irreversible activation of oncogenic KRas^{G12D} driven by the *kras* promoter when it inhales adenovirus expressing Cre recombinase.

This causes multifocal lung tumours to occur within 18 weeks. Shut down of Myc transactivation using transgenic Omomyc expression caused profound tumour reduction within 3 days and mice become overtly tumour free after 28 days [45]. Reassuringly murine tissue integrity was maintained with no major unexpected toxicities emerging. Considering neuroblastoma, treatments directed at MYCN are particularly appealing as genetic mouse models with MYCN targeted to neural crest tissue develop tumours which are similar to neuroblastomas [48].

3.2. MYCN in neuroblastoma

Double minutes and homogeneously staining regions are the cytogenetic hallmarks of genomic amplification in malignancies. Neuroblastoma karyotypes frequently have these cytogenetic markers and MYCN amplifications are often found [49]. Tumours that have an aggressive phenotype frequently have amplification of the MYCN oncogene and amplification of MYCN is correlated with the extent of disease at diagnosis [50]. Correlative International Neuroblastoma Staging System (INSS) stage at diagnosis and the respective frequency of MYCN amplification described by data from the Children's Oncology Group Statistical Office are; Stage 1, 3%; Stage 2a/2b, 4%; Stage 3, 25%; Stage 4, 32% and Stage 4s, 8% (Wendy, London; John, Maris, Principles and Practice of Paediatric Oncology, page 890). Amplification of MYCN is normally detected by interphase FISH with a usual increase in copy number of 50 to 400 copies in neuroblastomas and a cut off of 4 times the normal copy number being the definition of MYCN amplification by many pathologists. Other genes can be co-amplified with MYCN [51, 52]. Also comparative genomic hybridisation of tumours arising in a transgenic mouse model that overexpresses MYCN in neuroectodermal cells, found losses and gains of at least seven chromosomal regions. These were syntenic with comparable abnormalities detected in human neuroblastomas [52].

NVP-BEZ235 a dual inhibitor of the phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway decreases levels of MYCN protein and suppresses tumour proliferation and angiogenesis in neuroblastomas [53]. This partly arises because MYCN in tumour cells contributes to paracrine signalling between tumour cells and endothelial cells. It has previously been shown that blocking PI3K/mTOR causes destabilisation of MYCN protein with reduced VEGF secretion and inhibition of progression of neuroblastomas in murine models [54]. A study published in *Science Translational Medicine* in 2012 by Chantry and colleagues provided new information as to the separate 'intrinsic' and 'paracrine' including 'anti-angiogenic' mediated anti-tumour effects of inhibiting PI3K/mTOR signalling in neuroblastomas using NVP-BEZ235. Improved survival was seen in two mouse models, the first a xenograft tumour derived from a patient (MYCN-amplified human orthoptic xenograft) and the second a MYCN dependent transgenic model (transgenic for TH-MYCN; that recapitulates a MYCN amplified tumour arising in an autochthonous site) in which MYCN causes spontaneous tumour formation in mice. In both models there were reductions in tumour growth without tumour regression. This was attributed to a reduction in proliferation of neuroblasts and decreased tumour vascular density. PI3K inhibition caused de-repression of GSK3 β with consequential Thr58 phosphorylation and destabilisation of MYCN with a remarkable reduction in MYCN levels in tumours treated with NVP-BEZ235.

To establish tumour cell autonomous effects of NVP-BEZ235 on MYCN degradation, HUWE1 knockdown tumour cells (deficient in PI3K/mTOR-mediated MYCN proteolysis; a Thr58 mutant MYCN) were used to establish orthoptic xenograft models. It was demonstrated that HUWE1 were resistant to the anti-angiogenic effects of NVP-BEZ235 showing that MYCN was a critical target in vivo and part of the anti-angiogenic effect is a consequence of the transcription regulatory function of MYCN. It is possible that NVP-BEZ235 may emerge as a new therapeutic choice for the treatment of neuroblastoma.

4. ALK and MYCN

4.1. ALK and MYC co-operating neighbours

ALK and MYCN are the only established oncogenes in neuroblastoma [55, 56]. ALK mutations occur at an equal frequency in both low and high risk neuroblastomas and within all genomic subtypes which is suggestive that mutations within the ALK gene are not the only molecular aberrations that drives oncogenesis in this disease [57]. ALKF1174L mutated neuroblastomas are more common within the high risk neublastoma category [10]. In neuroblastoma cell line studies overexpression of wild type or mutated ALK stimulates the transcription of MYCN and the concurrent expression of MYCN and activated ALK increases the in vitro transformation of NIH3T3 cells [58].

A series of experiments on pathogenic cooperation between ALK and MYCN were conducted using a transgenic zebrafish model of neuroblastoma in which MYCN induced tumour arose from a subpopulation of neuroblasts which migrate to the interrenal gland, the zebrafish equivalent of the adrenal medulla (50% of neuroblastomas in humans arise in the adrenal medulla) [59]. Sympathoadrenal precursors in the interrenal gland co-express the cholaminergic enzymes tyrosine hydroxylase and dopamine- β -hydroxylase as well as neuronal specific Hu proteins. In this model the dopamine- β -hydroxylase promoter was used to drive EGFR-MYCN expression. Using this experimental model it was found that 17.3% of MYCN induced zebrafish develop neuroblastomas. Transgenic zebrafish that expressed wild type ALK or that expressed F1174L mutant ALK did not develop neuroblastomas. Activated ALK accelerate the onset of neuroblastoma and increased the penetrance of MYCN-induced neuroblastoma with a 3 fold increase for fish co-expressing both MYCN and ALK F1174L compared to wild type or F1174L mutant ALK fish. The mechanism as to how neuroblastomas arise within the zebrafish model was also investigated. MYCN overexpressing transgenic fish has an increased number of Hu⁺ neuroblasts that fail to differentiate resulting in increased cell number with reduced numbers of chromaffin cells compared to controls at 3-5 weeks post fertilization (wpf). An apoptotic response significantly reduced the number of these Hu⁺ cells at the 5-7 wpf interval. In the presence of a cooperating activated ALK there is continuous accumulation of Hu⁺ neuroblasts with failure of differentiation but there is decreased apoptosis of high penetrance and transformed neuroblastoma. Overall it has been inferred that ALK mutant F1174L attenuates the sequential apoptotic response in MYCN transformed Hu⁺

neuroblasts constituting the 'second' hit when considering it as an oncogenic equivalent of the Knudson 'two hit' model.

Another study found that ALK regulates the initiation of MYCN transcription in neuroblastoma [58]. ALK (including wild type ALK and mutated variants) stimulated the transcription of MYCN mRNA by affecting the MYCN promoter in neuronal and neuroblastoma cell lines. Similarly the transcription of MYCN can be abrogated by using ALK inhibitors such as crizotinib or NVP-TAE684. A series of experiments found that ALK^{F1174L} or ALK^{R1275Q} mediated a marked transformation of NIH3T3 cells but MYCN alone or wild type ALK failed to initiate cellular transformation. Co-transfection of ALK^{1174L} concurrent with MYCN caused a 3 fold increase in transformation compared to activated ALK^{F1174L} alone. Results consistent with this finding were seen when ALK^{R1275Q} was expressed with MYCN. It was consistently noted that ALK^{F1175L} has a greater transforming potential than ALK^{R1275Q} [60]. Overall it appears that ALK drives the initiation of MYCN transcription. Concomitant expression of a constitutively active mutant ALK variant causes increased transformation and MYCN protein levels compared with expression of ALK^{F1174L}, ALK^{R1275Q} or MYCN alone. Trials of ALK inhibitors alone in neuroblastoma may not succeed as dysregulation of MYC is the main 'oncogenic driver' in the disease and initiation of MYCN transcription can occur by ways other than by the mutation or amplification of ALK.

5. Recent therapeutic advances in neuroblastoma and the promise of targeted therapies changing the treatment paradigm

The treatment of neuroblastoma varies according to risk group stratification and prognosis. Therefore prognosis needs to be considered first prior to contextualizing recent therapeutic advances.

5.1. Prognosis

Phenotypic and prognostic variation occurs in neuroblastoma as some clinical phenotypes spontaneously regress while other patients have rapidly progressive high risk disease in which despite intensive myeloablative chemotherapy relapses are common and almost invariably fatal [61,62]. Neuroblastoma prognosis can be subdivided into low risk, intermediate risk, high risk, and stage 4S disease [63]. The U.S. National Cancer Institute also has listed different subdivisions of neuroblastoma. Firstly neuroblastomas can be subdivided into three biologically discrete types of tumor. These categories can be used to sub-stratify patient prognosis but do not have treatment implications [64,65].

Type 1: Hyperdiploid, expression of TrkA neurotrophin receptor. Tends to spontaneously regress.

Type 2: Expression of TrkB neurotrophin receptor and its ligand. Additional copy of chromosome 17q, loss of heterozygosity 14q or 11q. Genome unstable.

Type 3: Gain chromosome 17q, loss of chromosome 1p, MYCN amplification.

There also is a risk assignment system in North America that has been used clinically by the Children’s Oncology Group in studies such as COG-9641 and COG-A3961 [66, 67]. It has categories of low, intermediate and high-risk based on INSS stage, age, and tumor biology. Tumor biology characteristics considered are International Neuroblastoma Pathologic Classification (INPC), MYCN status and tumor DNA index. The INPC system involves the evaluation of pre-treatment tissue for the amount of stromal development, the mitosis-karyorrhexis index and the degree of neuroblastic maturation [68,69,70,71]. Stage 4S disease though included in these studies is a particular case.

5.1.1. *Treatment advances in high risk neuroblastoma in the ‘ALK Era’*

In 2009 long term outcomes of 379 patients with high risk neuroblastoma (CCG-3891) all of whom received the same induction treatment (5 cycles’ cisplatin, doxorubicin, etoposide and cyclophosphamide plus surgery and received radiotherapy for residual local and metastatic disease) was reported [72]. Subsequent to induction patients were randomly assigned to consolidation with myeloablative chemotherapy, total body irradiation, and autologous purged bone marrow transplantation versus 3 cycles of intensive chemotherapy (cisplatin, etoposide, doxorubicin and ifosfamide). Of the participants that completed consolidation without disease progression, they were randomly assigned to no further therapy or 6 cycles of 13-cis retinoic acid (160mg/m²/d in 2 divided doses for 14 days every 28 days). Myeloablative therapy and autologous hematopoietic rescue had a significantly better 5 year event free (~30% versus ~19%) and overall survival compared with non-myeloablative chemotherapy. 13-cis-retinoic acid after consolidation independently lead to significantly improved overall survival. 5 year overall survival from time of second random assignment for patients who underwent both sequential randomisations is documented in table 1.

Treatment randomly assigned to	5-year overall survival
ABMT / 13-cis retinoic acid	59%+/-8%
ABMY / no 13-cis retinoic acid	41%+/-7%
Continuing chemotherapy / 13-cis retinoic acid	38%+/-7%
Chemotherapy / no 13-cis retinoic acid	36%+/-7%

Table 1. please add caption

In 2010 a new treatment advance was reported in high risk neuroblastoma involving ch14.18 a chimeric human-murine anti-GD2 monoclonal antibody that targets GD2 a disialoganglioside tumour associated antigen [73]. Patients that had a response to induction therapy and stem-cell transplantation were treated with immunotherapy (six cycles of isotretinoin and five concomitant cycles of ch14.18 in combination with alternating GM-CSF and interleukin-2). This regimen was found to be better than standard treatment (six cycles of isotretinoin) with a 2 year event free survival rate of 66% compared to 46% respectively, P=0.02.

High risk disease has a generic treatment paradigm of intensive chemotherapy to induce remission followed by surgery, radiotherapy and myeloablative chemotherapy. A presentation at the 2011 ASCO Annual meeting changed the treatment standard for high risk disease. The HR-NLB1 trial was a comparator trial between two high dose myeloablative chemotherapeutic regimens in high risk neuroblastoma. 563 children with stage IV disease (high risk distant metastatic disease or local disease; median age 3 years) received busulphan and myelphalan (281 patients) or a 3 drug chemotherapeutic combination of carboplatin, etoposide and melphalan (CEM; 282 patients). The 3 year event free survival was 49% versus 33% respectively. The 3 year overall survival was 60% versus 48% again favouring busulphan-melphalan over CEM. Busulphan-melphalan also had a lower rate of relapse 47% versus 60% [74].

5.1.2. Intermediate risk neuroblastoma advances in the 'ALK Era'

A phase 3 non randomized trial of newly diagnosed intermediate risk neuroblastoma without MYCN amplification was performed on 479 patients (323 patients had favourable biology tumours; 141 patients had tumours with unfavourable biology) [75]. Patients with favourable histopathology and hyperdiploidy received 4 cycles of chemotherapy (carboplatin, etoposide, cyclophosphamide and doxorubicin, administered at 3-week intervals) and patients with unfavourable features or an incomplete response received 8 cycles. The 3 year overall survival rate was ~96% with an overall survival rate of 98% for patients with favourable biology tumour and 93% for patients with unfavourable biology neuroblastomas. Using this biologic based risk assignment high rates of survival were preserved in intermediate risk disease with reduced doses and duration of chemotherapy compared to historic controls (e.g. Children's Oncology Group trial CCG-3881; overall survival INSS stage 4s, 92%; stage 4, 93%; stage 3, 100%)[76,77,78]. Recent years have seen advancements in high risk neuroblastoma involving myeloablative conditioning regimens, 13-cis retinoic acid and immunotherapeutic. However in intermediate risk disease the evolution of treatment involves preservation of treatment efficacy using a biologically defined stratification approach with a reduction in the patient exposure to chemotherapy.

5.1.3. What of ALK and MYC and targeted treatments?

MYC gene transcription can be diminished by targeting BET bromodomains using small molecular inhibitors of the BET family of chromatin adaptors [79]. Inhibition of BET bromodomain-promoter interactions with reduced MYC mRNA transcription and translation of MYC protein caused G1 cycle arrest with apoptosis in a diverse number of lymphoma and leukaemia cells. There was dysregulation of the MYC transcriptome including reactivation of the tumour suppressor p21. Treating xenograft models of Burkitt's lymphoma or acute myeloid leukaemia with a BET inhibitor demonstrated significant anti-tumour activity. Activation of the c-MYC gene is the *sine qua non* of Burkitt's lymphoma with the c-MYC locus at Chr. 8q24 involved in t(8;14)(q24;q32) epidemic Burkitt's lymphoma and other abnormalities involving the MYC gene in sporadic cases including a different t(8;14) translocation and point mutation of exon 2 of c-MYC. In neuroblastoma cell lines with MYCN amplification high

dose transient treatment with (+)-JQ1 (a small molecule enantiomer BET bromodomain inhibitor) caused transcriptional repression of MYCN.

Zhu and colleagues in the zebrafish model of neuroblastoma show that ALK F1174L attenuates MYCN induced apoptosis [59]. Given the previous experience with targeted therapeutics in other disease types and the emergence of drug resistance it has been contended that responses to crizotinib are unlikely to be durable [57]. As ALK and MYCN have collaborative roles with MYCN being the primary oncogenic driver it may be the case that some of the initial optimism pertaining to Crizotinib in neuroblastoma may not be fulfilled and that dual targeting of ALK and MYCN once technically feasible may revolutionise the outcome for many patients with neuroblastoma. In a fascinating caveat it is notable that in MYCN single-copy cases of neuroblastoma, increased MYCN mRNA and protein levels are paradoxically associated with a more favourable clinical phenotype. Gene expression profiling of 251 primary neuroblastomas identified a core set of MYCN/c-MYC target genes with a successive gradual increase in that target gene signature in localized non-amplified cases to stage 4s-non amplified followed by stage 4-non-amplified and finally MYCN amplified cases. High expression of the MYCN/c-MYC gene signature identified patients with poor overall survival independent of some of the usual clinico-pathologic variables such as age at diagnosis (> or equal 1.5 years; stage 4 versus stages 1,2,3, and 4s and amplified MYCN) [80]. It is apparent that MYCN's role in the quite heterogenous disease neuroblastoma is complex and the development of targeted therapeutics in neuroblastoma needs to appreciate these complexities. Of course treatments that target MYCN remain therapeutic lacunae to be filled. A final ancillary comment on ALK and MYC. German investigators have designed and performed experiments using JoMa1 which is a multipotent neural crest progenitor cell line that is kept in an undifferentiated but viable state by a tamoxifen activated c-Myc transgene (c-MycER^T) [81]. Expression of ALKF1174L in primary neuroblastomas caused in vitro growth of these cells independent of c-MycER^T activity and the in vivo growth of neuroblastoma like tumours. Tumorigenicity was further enhanced by serial transplantation and remained susceptible to NBT-272 a MYC inhibitor. Therefore it appears that targeting ALK alone may result in regression of neuroblastoma with the potential to further augment tumour regression by inhibition of MYC. Maybe new hope for children with neuroblastoma does not fully rely on neighbourly gene relations!

6. Conclusion

The improvement in outcome in neuroblastoma treatment in recent decades is a tremendous success however outcomes for high risk disease have changed little. The importance of the ALK gene in this disease and an established ALK inhibitor already being used in other types of cancer offers new hope for improving outcomes. Therapeutic targeting of Myc is a long aspired for hope in the wider field of oncology which may be realised. Chromosomal proximity of genes such as topoisomerase II and Her-2 in breast cancer has been a topical subject but aside from the concept of co-amplification of genes as in the breast cancer 'topo II-Her2' paradigm ALK and Myc interact in many nuanced and therapeutically exploitable ways with the prospect of many advances still to come.

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