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The Role of Genetic and Epigenetic Alterations in Neuroblastoma Disease Pathogenesis

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TABLE OF CONTENTS

1 ABSTRACT

2 INTRODUCTION

3 CHROMOSOMAL ABERRATIONS

3.1 DNA Ploidy
3.2 Amplification of MYCN Oncogene
3.3 Chromosome 1p Deletions
3.4 Loss of Chromosome 11q
3.5 17 q Gains
3.6 Other Imbalances

4 Genetic Mutations

4.1 Familial Neuroblastoma
4.2 Sporadic neuroblastoma

5 mRNA Signatures

6 Non-coding RNA.

6.1 miRNAs
6.1.1 Copy Number Gains and Losses
6.1.2 Activators and Repressors of microRNA Transcription
6.2 Long non-coding RNAs

7 Conclusions

1 ABSTRACT

Neuroblastoma is a highly heterogeneous tumor accounting for 15% of all pediatric cancer deaths. Clinical behavior ranges from the spontaneous regression of localized, asymptomatic tumors, as well as metastasized tumors in infants, to rapid progression and resistance to therapy. Genomic amplification of the MYCN oncogene has been used to predict outcome in neuroblastoma for over 30 years, however, recent methodological advances including miRNA and mRNA profiling, comparative genomic hybridization (array-CGH), and whole-genome sequencing have enabled the detailed analysis of the neuroblastoma genome, leading to the identification of new prognostic markers and better patient stratification. In this review we will describe the main genetic factors responsible for these diverse clinical phenotypes in neuroblastoma, the chronology of their discovery, and the impact on patient prognosis.

2 INTRODUCTION

Neuroblastoma is a highly malignant pediatric cancer derived from precursor or immature cells of the sympathetic nervous system. Despite the relatively low incident level, (6-10 children per million) [1, 2] approximately 15% of all childhood cancer deaths can be attributed to the disease [3]. Long since recognized as a genetically complex form of cancer, neuroblastoma displays profound genetic heterogeneity (Figure 1). As a result, strikingly different outcomes are observed across tumor subtypes. These range from spontaneous regression without therapy (developing into a benign ganglioneuroma), to rapid progression and death due to disease. It is clear therefore, that in order to combat neuroblastoma, we must understand the genetics of the disease.

The significance of *MYCN* amplification (MNA) in neuroblastoma pathogenesis was first established in the early 1980's with its association with high risk tumors and poor patient survival [4]. Since that time, multiple recurrent genetic alterations have been associated with neuroblastoma, including whole chromosome gains and a large number of large-scale chromosome imbalances, such as loss of heterozygosity at chromosome arms 1p, 3p, 14q and 11q, unbalanced gain of 1q, 11p and 17q and numerous mutations in key genes such as *ALK*, *PHOX2B* and *PTPRD* [5-9].

Numerous studies have now demonstrated that genomic and transcriptomic profiles can be predictive clinical disease course, so that a combination of mRNA, miRNA and arrayCGH are now being used to better define prognostic signatures and may provide insight into the molecular basis of clinical heterogeneity [10-16]. This progress is somewhat reflected in the International Neuroblastoma Risk Group (INRG) staging system which takes into account both clinical characteristics and tumor biology to identify clinical risk groups with statistically different event-free survival rates [17]. Independently prognostic baseline characteristics included

in this system are patient age, disease stage, histology, grade of differentiation, DNA index, *MYCN* amplification status, and the presence of copy number aberrations at chromosome arm 11q.

In this review we will discuss the key genetic factors contributing to neuroblastoma as identified over the past 30 years, and the significance of such in relation to improved understanding of neuroblastoma predisposition in both familial and sporadic cases.

3 CHROMOSOMAL ABERRATIONS

The key to elucidating the means by which chromosomal aberrations reduce overall survival is to identify oncogenes and tumor suppressor genes located in the regions of alteration. Here we take a look at some of the most frequent aberrations found in neuroblastoma tumors, and the key protein-coding genes located at cancer-associated genomic regions (CAGRs) or in fragile sites.

3.1 DNA Ploidy

Generally, tumors from patients with low stage disease are hyperdiploid or near-triploid and have few, if any, structural aberrations [18]. This genetic subtype of tumor is frequent in patients of less than 1 year of age, where tumors are localized and have good prognosis [19]. Although ploidy can be predictive of outcome in infants, the prognostic significance of ploidy is lost for patients older than 1-2 years [20], probably because chromosomal aberrations in diploid tumors have contributed to the deregulation of cancer-related pathways.

Many neuroblastoma tumors display DNA diploid status and bear partial gains, losses, amplifications or other structural chromosome aberrations. Recurrent structural chromosomal alterations commonly associated with advanced stage of disease and poor outcome in neuroblastoma include *MYCN* amplification, deletion of chromosome arms 1p, 3p, 4p and 11q, and gain of chromosome arm 17q (For review see [21]). In addition, a recent INRG report on non-MYCN amplified tumors determined that it is not single genetic markers, but the overall segmental genomic profile of tumors that adds information to patient prognosis [22].

3.2 Amplification of MYCN Oncogene

Amplification of the *MYCN* gene, mapping to 2p24.1 (Figure 2), is present in approximately 20% of neuroblastoma tumors and since its discovery in the early 1980's remains one of the most important genetic abnormalities associated with advance stages of disease and a highly malignant phenotype [4, 23]. Numerous studies focusing on identifying the signalling pathways influenced by MYCN have established that high levels enhances the expression of numerous genes involved in cell proliferation, and also represses expression of differentiation- and apoptosis-related genes either in a direct or indirect fashion [24-26]. Targets directly induced by MYCN include the high mobility group A (*HMGA1*) [27], the minichromosome maintenance complex component 7 (*MCM7*) [28], the Mdm2-p53 binding protein homolog (*MDM2*) [29], p53 [30], and the multidrug resistance-associated protein *MRP1* [31].

Perhaps most significantly, the overall impact of MYCN was revealed in an early study by Weiss *et al.*, confirming over-expression of *MYCN* alone was sufficient to initiate neuroblastoma formation in mice [32]. Despite this, *MYCN* status cannot predict all cases of poor survival in neuroblastoma, and 80% of neuroblastomas do not display *MYCN* amplification. Several studies have reported over-expression of *MYCN* in the absence of amplification [33, 34]. A recent study revealed a functional 157-gene signature in neuroblastoma consisting of relevant genes that are regulated by MYCN and predictive of outcome. Interestingly, a sub-group of the tumors displaying this signature and poor outcome did not have *MYCN* amplification or high *MYCN* mRNA levels, but high nuclear MYCN protein levels [35]. This suggests that the aggressive phenotype of *MYCN* might not only be associated with *MYCN* copy numbers, but with other signals that regulate *MYCN* expression, such as are RNA binding proteins (RBP) and microRNAs, which we will discuss in more detail later.

3.3 Chromosome 1p Deletions

Loss of heterozygosity (LOH) of the short arm of chromosome 1p is found in 20-35% of neuroblastoma tumors [36, 37]. This aberration is frequently associated with amplification of *MYCN*, and is found approximately in 70% of aggressive neuroblastomas [38], and it has been reported that 1p LOH is independently associated with poor outcome [39].

Pinpointing candidate tumor suppressor genes in the 1p LOH genetic subtype was aided by the identification of the shortest region of consistent heterozygous deletion (spanning 261kb) at 1p36.3 [40-42]. One of the first genes identified was the chromodomain helicase DNA binding domain 5 (*CHD5*), mapping to 1p36.31 [43]. Very low levels of CDH5 were observed in 137 neuroblastoma primary tumors and cell lines, and that low expression of CHD5 was highly correlated with 1p LOH, *MYCN* amplification, advanced stage, and unfavorable histology. Consistently, Fujita *et al.*, reported that tumor growth was inhibited in mice over-expressing *CHD5* [44]. In addition, their data strongly suggested that inactivation of the second allele of *CHD5* in neuroblastoma occurs by means of epigenetic silencing. Based on the positive correlation between 1p LOH and *MYCN* amplification found in neuroblastoma tumors, the authors suggested that *CHD5* promoter methylation could be a MYCN mediated effect [45].

Recently, *CAMTA*1, a transcription factor mapping to 1p36, was also identified as a tumor suppressor gene in neuroblastoma. Multivariate survival analysis, based on *CAMTA1* mRNA expression profiling data in a cohort of 251 neuroblastoma tumors, confirmed that low *CAMTA1* was a predictor of poor clinical outcome independently of *MYCN* status, 1p LOH, and age of the patient at diagnosis [46]. In a follow up study, transcriptome analysis using a *CAMTA1*-inducible cell model revealed that expression of *CAMTA1* induces the transcription of genes involved in neuronal differentiation, and inhibits genes related to cell proliferation. In addition, subcutaneous inoculation of athymic nude mice with *CAMTA1*-inducible neuroblastoma cells resulted in a significant reduction of the tumors, demonstrating a role for *CAMTA1* as a tumor suppressor in an *in vivo* model.[47].

Zinc-finger transcription factor *CASZ1*, located on 1p36.22, has also been suggested to play a role in cell differentiation. A study by Lie *et al.*, reported low expression of *CASZ1* mRNA was found in 77% of neuroblastomas of patients older than 18 months (n=59), and significantly associated with decreased overall

survival [48]. Low *CASZ1* mRNA levels, as measured by quantitative real-time PCR, were associated with neuroblastomas with a poor differentiated histopathology and significantly correlated with increased age (≥ 18 months), 1p LOH, *MYCN* amplification and advanced disease. Consistently, restoration of *CASZ1* in neuroblastoma cell lines induced cell differentiation, enhanced cell adhesion, and suppressed cell growth [48]. Subsequent studies demonstrated that silencing of the second allele of *CASZ1* was mediated by the aberrant up-regulation of the polycomb protein histone methyltransferse *EZH2*, which regulates differentiation in many tissues [49].

Other strong candidate 1p tumor suppressor genes include the ubiquitination factor E4B (*UBE4B*) and apoptosis-inducing, TAF9-like Domain 1 (*APITD1*). The expression of *UBE4B*, a gene implicated in the ubiquitin/proteasome pathway, is markedly decreased in high-stage/poor-prognosis tumors compared to low-stage/favourable-prognosis tumors [50]. In functional studies *APITD1*, which is also lowly expressed in neuroblastoma, reduced cell growth in the neuroblastoma cell lines SK-N-AS and SK-N-BE [51].

3.4 Loss of Chromosome 11q

Another common structural chromosome aberration associated with aggressive clinical behaviour is 11q LOH, occurring in approximately 40-45% of cases [52]. Although inversely correlated with *MYCN* amplification [52-55], a small sub-set of tumors display both an 11q LOH and *MYCN* amplification. Numerous studies suggest that *MYCN* amplified and 11q LOH represent 2 distinct subtypes of neuroblastoma tumors, both of which can be associated with poor clinical outcome [52, 56, 57]. As a result, in 2009, aberrations of chromosome 11q were included in the international neuroblastoma risk group (INRG) classification system [17].

Therefore, identifying genes on the 11q chromosome that contribute to neuroblastoma aggressiveness is crucial to understanding the pathways deregulated in these tumors. However, in spite of the intensive effort, only a few genes have been identified to date. A study by Carén *et al*., reported that in tumors with 11q LOH the frequency of segmental aberrations was significantly higher than in MNA tumors as determined using high-density SNP microarrays. This fact was explained in part by the loss of the *H2AFX* gene, located in the 11q23.3 deleted region [58]. This gene has been shown to play a role in genomic stability modification, and enhanced susceptibility to cancer in mice [58, 59].

Other studies identified Cell Adhesion Molecule 1 (*CADM1*), which transcribes a cellular adhesion protein involved in neural cell development, as a candidate tumor suppressor gene in the 11q23 deleted region. *CADM1* was significantly down-regulated in tumors with 11q LOH relative to 11q diploid tumors, and significantly associated with advance stage of disease and poor survival [60, 61]. Over-expression of *CADM1* revealed a significant inhibition of cell proliferation and colony forming ability in a panel of 4 different cell lines, demonstrating that *CADM1* expression attenuates the malignant phenotype in cultured cells. No evidence for inactivating mutations of *CADM1*, or hypermethylation of its promoter was found, suggesting that other mechanisms such as haplosufficiency or post-transcriptional regulators of gene expression may be involved in the modulation of *CADM1* expression. [60, 62].

Constitutional rearrangements of 11q have also been reported in patients with neuroblastoma [63, 64], indicating that aberrations in 11q genes may also be involved in development of neuroblastoma. Although 11q LOH remains strongly associated with poor outcome in neuroblastoma, more recent work now demonstrates that both miRNA and mRNA expression profiles are more powerful predictors of clinical outcome than 11q status alone [14, 65].

3.5 17 q Gains

The most common aberration found in neuroblastoma tumors is the unbalanced gain of 17q (segment 17q21-qter), occurring in ~70% of tumors [66-68]. Frequently, this aberration is caused by unbalanced translocations of segment 17q21-qter and the distal part of chromosomes 1p or 11q [66, 69-74], though other chromosomes can also be involved in 17q gains [75]. Numerous studies have reported that 17q gain is significantly associated with advanced stage of disease, increased patient age, 1p LOH, 11q LOH, and *MYCN* amplification [68, 71, 75]. However, the independence of 17q gain as a prognostic factor is controversial. A study by Bown *et al*, investigated the prognostic independence of 17q gain by analyzing the 17q status and clinical data for 313 tumors from 6 different European institutes. Multivariate analysis including patient age, tumor stage, *MYCN* status and 1p LOH demonstrated that 17q gain was an independent prognostic factor. However, *MYCN* status and patient age were not predictive of survival in this model [68]. Contrary to this, Buckley *et al*., determined that 17q gain was not an independent predictor of poor survival [38], as did Spitz *et al*, who reported that when 17q gain, 11q LOH and *MYCN* status were included in a multivariate analysis, 17q gain was not a significant prognostic factor, while *MYCN* and 11q LOH were [76]. Whether an independent prognostic factor, or merely a modifying factor, identifying the gene aberrations caused by 17q unbalance will be crucial to fully understanding neuroblastoma progression.

3.6 Other Imbalances

In addition to these major genetic aberrations found in neuroblastoma, there are other recurrent imbalances that could also be important, such as gain of chromosomes 1q, 2p, 7q, 9p and 11p, or loss of 3p, 4p, 14 q, 16p and 19q [77]. However, the biological significance of these aberrations and the genes contributing to neuroblastoma pathogenesis remains elusive. Figure 2 displays a summary of the aberrations identified across the genome on 160 primary neuroblastoma tumors, as determined in a study by Buckley *et al.* [78].

4 Genetic Mutations

Mutations are one of the several alterations at genome level that can provoke malignant transformation or tumor progression, and heavily contribute to neuroblastoma clinical heterogeneity. Table 1 summarizes published clinical studies focused on mutations identified in neuroblastoma.

4.1 Familial Neuroblastoma

Familial neuroblastoma is a rare event, as it only accounts for 1-2% of cases. Inheritance seems to follow an autosomal dominant pattern with incomplete penetrance [79]. As in sporadic neuroblastoma, the familial cases also display a significant clinical heterogeneity ranging from tumors that spontaneously regress to tumors that rapidly metastasize [80]. This suggests that the different outcomes could be linked to differences in additional somatically acquired mutations. Inherited mutations in the homeodomain transcription factor paired-like homeobox 2B *(PHOX2B)* and the anaplastic lymphoma kinase (*ALK*), have been reported to predispose to familial neuroblastoma [5-7]. PHOX2B is a homeodomain-containing protein which plays an essential role during early development promoting neuron formation and differentiation [81]. Missense or frame-shift mutations in the homedomain of *PHOX2B* were described in a rare subset of neuroblastomas with congenital central hypoventilation syndrome (CCHS). However, this mutation was reported to occur in only 6.4% of familial neuroblastomas [82]. In addition, another study screening 237 sporadic neuroblastoma tumors, revealed that mutations in *PHOX2B* were found only in ~2% of the cases [83]. This suggests that, although *PHOX2B* could give selective advantages to tumor cells, it is likely not sufficient to drive neuroblastoma pathogenesis.

Contrary to this, activating mutations of *ALK* are found in the majority of familial cases of neuroblastoma, as well as in 12.4% of high risk sporadic neuroblastomas [6]. Although the role of ALK during normal development remains to be elucidated, it is clear that activating mutations in *ALK* promote oncogenesis in neuroblastoma and in other type of cancers [7, 84]. DNA amplification and protein over-expression, as well as activating point mutations of *ALK,* have been described in neuroblastomas [85] . A recent study demonstrated that *ALK* (F1174L), which is the most frequent and aggressive *ALK* mutation, was sufficient to promote neuroblastoma development in mice. In addition, when *ALK* (F1174L) and *MYCN* were co-expressed, a synergic effect was displayed in tumor development. Interestingly, these tumors had minimal chromosomal aberrations, suggesting that these two genes are sufficient to drive neuroblastoma formation [86].

4.2 Sporadic neuroblastoma

Almost 98 % of neuroblastoma cases represent sporadically arisen tumors. Sporadic neuroblastoma is driven by multiple, low frequency polymorphisms. Advances in genome sequencing technologies have allowed genome wide association studies (GWAS) resulting in the identification of risk polymorphisms in a number of large, independent studies [87-89]. *Maris et al.* genotyped genomic DNA from 1032 NB patients and 2043 control subjects [89]. Association analyses of chromosome 6p22 SNPs were performed for 883 patients where complete clinical data were available. The authors identified three repeated common SNPs within two overlapping genes, *FLJ22536* and *FLJ44180*, a non-coding RNA gene, at 6p22 showing genome-wide significance for association with sporadic neuroblastoma. Homozygosity for any of these risk alleles was significantly associated with high-risk features including metastatic disease, *MYCN* amplification, and lower patient survival. The same dataset also identified 6 SNPs at 2q35 located within introns 1, 3, and 4 of the BRCA1-associated RING domain-1 (*BARD1*) gene [87]. Apparently, *BARD1* plays an important role in the tumor suppressor function of *BRCA1*, a hereditary breast and ovarian cancer susceptibility gene, and pathogenic *BRCA1* mutations have been shown to impede with the heterodimerization of BRCA1 and BARD1[90]. An expansion of the original GWAS cohort

included 2,251 neuroblastoma patients and 6,097 controls [88], documented an additional predisposition locus at 11p15, within the LIM domain only 1 (*LMO1*) gene, a transcriptional regulator. Duplication of the *LMO1* locus was reported to occur in 12% primary neuroblastoma tumors and was associated with more advanced disease as well as two SNPs. Supplementary *in vitro* studies demonstrated that siRNA knock down of *LMO1* inhibits cell growth, and *LMO1* over-expression enhances cell proliferation, supporting the role of *LMO1* as an oncogene involved in the pathogenesis of neuroblastoma.

Another group used a whole-genome paired-end sequencing platform to identify mutated genes associated with sporadic neuroblastoma in 87 untreated primary neuroblastomas, with an additional validation by SNP arrays of 52 sequenced tumors [9]. The sequencing data identified very few recurrent somatic mutations. In contrast, novel genetic defects were identified in high-risk tumors. First, massive genomic rearrangements, known as chromothripsis [91], were observed in 18% of high –, but not low-stage tumors. Chromothripsis was significantly associated with poor-survival, *MYCN* amplification, 1p LOH, and affected genes involved in neuroblastoma pathogenesis. In addition, structural aberrations in neuritogenesis genes were also found in high-stage tumors without *MYCN* amplification, which could explain the aggressive behavior of this subtype of tumors.

Very recently, over-expression of the RNA binding protein LIN28B was reported to be associated with the presence of a SNP within an intron of the *LIN28B* gene in neuroblastoma [92]. *LIN28B* mRNA expression was significantly higher in cell lines homozygous for the risk allele compared to heterozygous cell lines, and over-expression of *LIN28B* was significantly associated with poor survival. However, none of the 12 neuroblastoma cell lines tested was homozygous for the protective allele. In addition, cell lines homozygous for the SNP of *LIN28B* had higher levels of *MYCN* expression. In addition, Molenaar *et al*., reported that high level amplifications of the 6q21 region, where LIN28B maps to, occur at low frequency in neuroblastoma tumors [93]. Regardless of the mechanism leading to *LIN28B* over-expression, Molenaar *et al*., demonstrated for first time that *LIN28B* over-expression was predictive of survival independently of *MYCN* and *ALK* status, patient age or tumor stage. Most importantly, this study demonstrated that transgenic mice expressing Lin28b in the neural crest developed tumors characterized for a histology and location similar to human neuroblastomas. *LIN28B* has therefore emerged as a new oncogene in neuroblastoma and a novel therapeutic target. The mechanism of action of *LIN28B* in neuroblastoma will be further discussed below.

5 mRNA Signatures

In an effort to further improve the risk estimation of neuroblastoma patients; many groups have identified mRNA expression patterns that allow more detailed subset classification and prediction of outcome. By comparing gene expression patterns in Stage 4S vs Stage 4 tumors (*MYCN* non-amplified) using serial analysis of gene expression (SAGE) Fischer *et al.,* found a predominance of genes involved in neuronal differentiation in 4S tumors compared to Stage 4 tumors, despite the poorly differentiated histopathology observed in both subtypes. They validated the expression levels of 18 genes by qPCR and used the combined analysis of these transcripts to show that patients with a favourable gene signature displayed significantly better EFS than those

with an unfavourable gene signature [94]. Asgharzadeh *et al*., also carried out a study on *MYCN* non-amplified tumors, and identified a 55 genes signature capable of stratifying clinically identical high-risk tumors into sub-groups with different outcomes [10].

In a retrospective SIOPEN/COG/GPOH study, Vermeulen *et al*., identified a 59-gene expression signature which could independently distinguish between patients with respect to progression –free survival (PFS) and overall survival (OS) in the SIOPEN and GPOH cohort [11]. The signature was also tested within each SIOPEN treatment protocol and found to accurately identify patients with risk of progression or relapse. A 236 tumor cohort from the Children's Oncology Group (COG) was used as a validation set, and found that the signature was the most significant variable for prediction. Forty-two genes in the 59-gene signature were also found to be present in at least 2 out of 4 published NB expression studies profiling high risk and low risk sub-groups [95-98]. The authors then used these 42 genes to define a cross-platform signature that was validated on four independent sets [12]. An additional study found 3 genes from the 42-gene classifier also contributed to a 6-gene signature (*MYCN, ALK, BIRC5, CCND1, NTRK1 and PHOX2B*) that distinguishes between four molecular subgroups of neuroblastoma [99]. In addition to the delineation of the established sub-groups of Type 1 (low risk, high *NTRK1*), Type 2A (intermediate risk, 11q-) and Type 2B (high risk, MNA), the authors identified a novel fourth sub-group using this 6-gene signature, consisting of high-stage 11q- tumors displaying low *MYCN* and *ALK* expression. The identification of this fourth sub-group is in concordance with the findings of Fischer *et al*., and Buckley *et al*., that 11q- tumors can be subdivided into two groups with distinguishing gene and miRNA expression profiles, predictive of outcome [37, 38].

6 Non-coding RNA.

6.1 miRNAs

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, small non-coding RNA molecules, approximately 22 nucleotides in length. They function as post-transcriptional gene regulators through targeting regions of partial sequence complementarity mainly at the 3'UTR (untranslated region) of the target mRNA resulting in the degradation of the mRNA or inhibition of protein translation [100]. This partial complementarity allows miRNAs to regulate multiple mRNA sequences. At the same time, a single mRNA can be regulated by multiple different miRNAs, resulting in a sophisticated gene regulatory network. MiRNAs are known to regulate oncogenes, tumor suppressor genes, genes involved in cell cycle control, cell migration, apoptosis and angiogenesis. Subsequently, altered miRNA profiles are found in several human diseases, as well as in many forms of cancer [101-104]. In fact, select miRNA signatures can classify multiple cancers more accurately than data from ~16,000 mRNAs [105].

In 2007, Chen *et al*. determined that many miRNAs were differentially expressed in different genomic subtypes of neuroblastoma, and that those miRNA profiles were correlated with prognosis, differentiation and apoptosis [15]. A continuation of this work was published in 2009 when an extended tumor cohort was analyzed for expression of 450 miRNA loci [16]. This study highlighted that over-expression of the MYCN transcription factor as well as large-scale chromosomal imbalances had contributed to the widespread dysregulation of miRNA expression in neuroblastoma tumors. Importantly, a miRNA expression signature predictive of clinical

outcome was identified, emphasizing the potential for miRNA mediated diagnostics and therapeutics. Consistent with these results, Schulte *et al*. reported that 7 miRNAs (miR-92, miR-106a, miR-let7b, miR-17-5p, miR-93, miR-99 and miR-221) were up-regulated by MYCN in neuroblastoma, and demonstrated that miR-221 was directly induced by MYCN *in vitro* [106] .

Emerging functional studies have established that miRNAs regulate important genes involved in neuroblastoma disease. For example, over-expression of the miR-17-5p-92 cluster promotes tumorigenesis by regulating the pro-apoptotic gene *BIM*, the cell cycle regulator p21, transcription factor *TGF-β* and the tumor suppressor *Dickkopf* [107-109]. Other miRNAs can act as tumor suppressor miRNAs, such as let-7 and miR-101 which directly regulate *MYCN* expression [93, 110], or have an anti-tumorigenic effect, such as the pro-apoptotic miR-34a [111-113], the anti-invasive miR-335 [114], novel tumor suppressor miR-542-5p [115] and several differentiation-related microRNA (miR-125b, miR-10a and miR-10b [116-118]). Other miRNAs have been shown to be related to drug sensitivity in neuroblastoma, such as miR-204 [119]. The list of specific miRNAs validated as contributors to neuroblastoma pathogenesis (Table 2) is ever expanding, but, what are the mechanisms underlying their deregulation in neuroblastoma?

6.2 Alteration of miRNA Expression: Mechanisms Involved.

Altered expression of miRNAs can be caused by multiple mechanisms, including aberrations in DNA copy number, altered transcriptional activators/repressors, aberrant DNA methylation, or defects of the proteins involved in the miRNA biogenesis machinery and in the post-transcriptional regulation of miRNA expression. The elucidation of the mechanisms involved in miRNA deregulation is required, not only to better understand the role miRNAs play in the development of the disease, but also may help us to identify new therapeutic targets.

6.2.1 COPY NUMBER GAINS AND LOSSES

As already mentioned, alterations in miRNA expression can be altered by DNA gains and losses. However, in addition to simple dosage effects, imbalance of miRNAs leads to altered expression of their target genes resulting in significant dysregulation throughout the genome. The finding that miRNAs are frequently located at fragile sites and genomic regions involved in cancer further implicates their involvement with malignant diseases [120].

Integrated analysis of miRNA expression profiling together with oligonucleotide arrayCGH revealed that many of the recurrent large-scale chromosomal imbalances in neuroblastoma tumors, including loss of 1p, 3p, 11q and 14q, along with gain of 1q and 17q, have a major impact upon miRNA expression [16]. This same study identified a 15-miRNA signature predictive of survival in neuroblastoma. In a subsequent study it was demonstrated that 11q LOH tumors could be split into distinct subtypes using a miRNA signature that differed significantly in clinical outcome and the overall frequency of large-scale genomic imbalances, with the poor survival group having more imbalances [78]. However, this study also found cases where miRNA expression was inversely related to the genomic imbalance, that is, miRNAs were under-expressed in spite of mapping to a

region of DNA copy number gain. This strongly suggests that alternative mechanisms can in some instances counteract the effects of DNA dosage.

In neuroblastoma, 1p LOH is frequently associated with *MYCN* amplification and poor outcome [38, 39]. One of the first tumor suppressor miRNAs identified as mapping to the shortest region of overlap was miR-34a, mapping to chromosome 1p36. Initial studies demonstrated that miR-34a could induce apoptosis in neuroblastoma cells [121], a function explained somewhat by the fact that miR-34a is directly activated by p53 [122] and soon after, *MYCN* was also identified as a direct target of miR-34a [123]. MiR-34a functions as a tumor suppressor miRNA by inducing apoptosis of neuroblastoma cells [121, 123, 124]. The multi-gene targeting nature of miR-34a is well documented, with target transcripts including *MYCN, BCL2, SIRT1, NOTCH1, JAG1, CCND1, CDK6*, and *E2F3* [113, 121, 123, 125-128]. It is no surprise then that the potential of miR-34a to act as a novel therapeutic in neuroblastoma was further explored. Targeted delivery of a miR-34a encapsulated anti-GD(2)-nanoparticles was accomplished in a neuroblastoma mouse model and confirmed miR-34a as an effective inhibitor of neuroblastoma tumor growth in vivo [112, 129].

6.2.2 ACTIVATORS AND REPRESSORS OF MICRORNA TRANSCRIPTION

6.2.2.1 MYCN and C-MYC

The MYCN transcription factor exerts regulatory control over the activation or repression of a large group of oncogenic and tumor suppressor miRNAs in neuroblastoma [16, 130]. Activation or repression of miRNAs is thought to occur by direct binding of MYCN to the proximal region of miRNAs loci. Amplification of the *MYCN* oncogene was shown to contribute to the widespread miRNA deregulation in neuroblastoma tumors, with miRNA profiles correlating with clinical outcome [16, 130]. These two independent studies reported that, in the majority of cases, miRNAs were being down-regulated in MNA tumors. This posed the questions: is MYCN directly regulating these miRNAs, or is it the result of a secondary event?, and What role do these differentially expressed miRNAs play in neuroblastoma cells?

MYCN binding to a large number of promoters and CpG islands was identified in neuroblastoma cell lines [131]. Chip-chip analysis of MYCN binding sites in neuroblastoma cell lines expressing both high and low levels of MYCN protein were performed in order to characterize the binding behavior of MYCN in these states. The analysis revealed that MYCN preferentially binds to non-canonical E-box sequence of CATGTG and to additional motifs when it is over-expressed; indicating that MYCN binding becomes less specific when highly abundant. However, to date the promoter regions of few miRNAs have been identified [132], and validation of this mechanism will not be possible until the exact promoter region of each miRNA is determined. Recently, the repression of a panel of miRNAs by MYCN binding was confirmed by CHIP-sequencing analysis in neuroblastoma cell lines. Of these miRNAs, miR-591 had tumor suppressive effects in an orthotopic neuroblastoma xenograft, while miR-558 revealed an oncogenic phenotype. This study reveals that MYCN can regulate the expression of both oncogenic and tumor suppressor miRNAs [133].

As our knowledge builds we have also become aware that miRNAs can play both pro-oncogenic and tumor suppressor functions depending on the environmental context. An interesting example is the miR-17-5p-93

polycistronic cluster. This cluster located in chromosome 13 (13q31.3 loci) encodes 7 mature miRNAs (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92) that play different roles in the cell. Of particular interest is miR-17-5p, which has been shown to have a pro-oncogenic function in several types of cancer [134-138], while also being reported to act as a tumor suppressor by targeting the oncogene E2F1 [139]. Fontana *et al* [107] established that this cluster was highly expressed in neuroblastoma cells over-expressing *MYCN* or having *MYCN* amplification, and verified MYCN binds directly to the 5' and 3´of the 17-5p cluster, promoting expression of these miRNAs. MiR-17-5p was found to enhance tumorigenesis by binding to the 3' UTR of *p21* and *BIM*, resulting in increased proliferation and inhibited apoptosis, respectively. A study by Mestdagh *et al*., in 2010 examined global protein response to up-regulation of miR-17-92 and found the *TGF-β* pathway is also suppressed in neuroblastoma upon miR-17-92 activation. Impaired TGF-β signaling subsequently contributes to poor prognosis in neuroblastoma patients [108]. More recently, the tumor suppressor *Dickkopf-3* (DKK3) was also validated as a direct target of miR-92a and miR-19b, both members of the miR-17-92 cluster [109].

According to the results of Bray *et al* and Mestagh *et al*. most of the differentially expressed miRNAs between MYCN amplified or MYCN single copy tumors were down-regulated [16, 130]. However, relatively few of the under-expressed miRNAs (n=4) appear to be directly regulated by binding of MYCN based on unbiased ChIP sequencing studies [133], indicating that many of these miRNAs are indirectly repressed.

6.2.2.2 p53

p53 is a tumor suppressor protein that plays a major role in the protection of genomic stability and prevention of tumor development by directly activating several genes, including miRNAs, which promote DNA repair, cell cycle arrest and apoptosis. Direct binding of p53 is responsible for the activation of the miR-34 family of miRNAs [140] which, as already explained, functions as a tumour suppressor miRNA in neuroblastoma. In addition to the miR-34 family, p53 also directly regulates the transcriptional expression of other miRNAs through direct binding to their promoter, such as miR-145, miR-107, miR-192 and miR-215 [141]. Although the role of these miRNAs in neuroblastoma remains to be elucidated, other studies suggest that these miRNAs act as tumor suppressors in other forms of cancer [142-144].

Inactivating mutations or deletions in the p53 gene are found in >50% of adult human cancers [145]. However, in neuroblastoma, this gene is seldom mutated, occurring in <2% of cases at diagnosis, and ~15% at relapse [146]. Nevertheless, inactivation of p53 can occur by an alternative mechanism in neuroblastoma tumors, for example, *MDM2* has been reported to act as a negative regulator of p53 expression [147]. Although MYCN can promote the expression of p53 [30], over-expression of *MDM2*, occurring in 29% of neuroblastomas, can counteract its effects, resulting in p53 inactivation and the aberrant expression of the p53-regulated genes [146, 148]. Taken together, this evidence suggests inactivation of p53 in neuroblastoma can cause the deregulation of both protein-coding genes and miRNAs, resulting in the deregulation neuroblastoma related pathways.

6.2.2.3 DNA Methylation

DNA methylation consist in the addition of a methyl group 5 of the cytosine within the dinucleotide CpG. Gene silencing can occur by aberrant hypermethylation of CpG islands, which are dense clusters of CPG dinucleotides often present in gene promoters. Neuroblastoma genome displays distinct patterns of DNA methylation which can be associated with different risk groups [149]. One of the first genes reported to be differentially methylated in neuroblastoma tumors was the tumor suppressor *RASSF1A,* located at 3p21.3 [150]. Inactivation of *RASSF1A* was observed to occur in 55% of a cohort of 67 neuroblastomas, suggesting that silencing of this tumor suppressor gene could contribute to neuroblastoma disease [150]. Another example of a commonly methylated and inactivated gene in neuroblastoma is *CASP8*. The *CASP8* gene plays an important role in the tumour necrosis factor-related apoptosis pathway [151]. An investigation of a cohort of 70 neuroblastoma tumour samples displayed 56% hypermethylation which was correlated to poor outcome in neuroblastoma [152]. In another study, involving clustering of a limited number of hypermethylated genes, *CASP8* was found to be methylated in 77% of the neuroblastoma cell lines investigated, further supporting the importance of the methylation status of this gene *in vitro* [153]. To date, more than 75 genes have been described as methylated in neuroblastoma tumors (for review see [154]), and more importantly, the methylation status of several genes have been shown to be associated with patient survival or neuroblastoma risk factors, such as, *MYCN* amplification, patient age and tumor stage [149, 151, 152, 155-157].

In neuroblastoma, all-trans retinoic acid (ATRA) treatment induces some neuroblastoma cell lines to differentiate, leading to profound changes in mRNA and miRNA expression [118]. In a study by Das *et al.*, DNA methylation changes were compared in SKNBE ATRA-treated versus untreated cells using methylated DNA immunoprecipitation applied to microarrays [158]. The authors identified a total of 402 gene promoter de-methylated following ATRA treatment, while only 88 genes became hypermethylated. The demethylation events were explained in part by the down-regulation of the methyltransferases DNTMT1 and DNTMT3 along with the up-regulation of endogenous miRNAs targeting them, such as miR-152 and miR-26a/b. The question is: are miRNAs epigenetically regulated?

Similar to protein coding genes, miRNAs are susceptible to epigenetic regulation. A recent study, compiling the methylation data available from several different neoplasms, revealed that in comparison to protein coding genes, miRNAs displayed a higher magnitude of methylation, with about 11.6% of all known miRNAs being methylated [159]. However, very few studies in this area have been reported in relation to neuroblastoma disease. Recently, Das et al. attempted to explore DNA methylation as a possible mechanism for the dysregulation of miRNA expression in neuroblastoma. In depth analysis of DNA methylation patterns in conjunction with miRNA and mRNA expression profiles in neuroblastoma clinical samples allowed to identify a large set of epigenetically regulated miRNAs with significantly enriched target sites in the 3′-UTRs of genes over-expressed in unfavorable tumor subtypes [160]. Notably, a high proportion of both the methylated miRNAs (42%) and their associated mRNA targets (56% of the highly redundantly targeted mRNAs) were highly associated with poor clinical outcome when under and over-expressed in tumors, respectively. The potential epigenetically-regulated miRNAs included well characterized tumour suppressor miRNAs in neuroblastoma such as some of the let-7 miRNAs, miR-29c, miR-101, miR-335, and miR-184. Importantly,

many of the genes targeted by this panel of miRNAs are known to play oncogenic roles in neuroblastoma, such as *AKT2, LIN28B, and CDK6,* suggesting that epigenetic silencing of miRNAs could contribute to the over-expression of oncogenes in neuroblastoma.

6.2.2.4 RNA Binding Proteins: LIN28B

A number of proteins that regulate miRNA processing have been described as key elements in defining the characteristic expression patterns of miRNAs in different cells or during disease. RNA binding proteins (RBP) can bind to primary or precursor miRNAs to regulate their expression. It has been determined that 14% of all human pri-miRNAs have terminal loops that are conserved throughout evolution, which may act as docks for RBPs regulating miRNA biogenesis [161]. Here, we focus on the RBP *LIN28B*, which has recently demonstrated to induce neuroblastoma development, and might represent a promising therapeutic target [93].

The RNA binding protein Lin28 was initially discovered in Caenorhabditis elegans as an important regulator of developmental timing. The mammalian homologues *Lin28* and *Lin28B*, are highly expressed in embryonic cells, and are responsible for maintaining the undifferentiated state of the cells [162]. *Lin28* is a target of the let-7 family of miRNAs [163, 164], and as the cells start to differentiate the let-7 levels are increased to down-regulate Lin28 expression [165, 166].

Recently, it was discovered that Lin28 blocks let-7 miRNA maturation by blocking let-7 processing at the level of DROSHA or DICER during miRNA biogenesis [167, 168]. Binding of LIN28B to the terminal loop of pre-let-7 induces the uridylation of let-7 precursor by recruiting the terminal uridylyl transferase (TUTases) to the pre-let-7 through a tetranucleotide sequence motif (GGAG), resulting in the addition of an oligouridine tail to the pre-let-7 [169-171]. Oligouridylation results in DICER blockage and degradation of the pre-miRNA by an unidentified nuclease. Lin28 can also repress DICER processing of the pri-let-7. Similarly, Lin28 recognizes the terminal loop of the pri-let-7 which inhibits DICER cleavage in vitro [164]. In addition to their role as negative regulators of the let-7 members, they are pluripotent stem cell factors, which together with three additional factors (Oct4, SOX2 and Nanog) are sufficient to reprogram somatic fibroblasts to become pluripotent stem cells [172]. The importance of LIN28 and LIN28B as regulators of the let-7 members emerged with studies demonstrating the up-regulation of LIN28 and LIN28B in several forms of cancer, including hepatocarcinoma, ovarian cancer, Wilm's tumor and chronic myeloid leukemia [173]. Over-expression of *LIN28* and *LIN28B* has also been associated with cellular transformation, and enhanced metastasis [173-175].

Very recently, two independent studies related LIN28B over-expression to neuroblastoma pathogenesis [92, 93]. Diskin *et al*., reported that SNPs in the *LIN28B* gene had contributed to the over-expression of *LIN28B* in neuroblastoma tumours. The mechanism by which *LIN28B* exerts its oncogenic roles was explained in the study by Molenaar *et al*. Consistent with the role of LIN28B being a negative regulator of let-7 expression, silencing of *LIN28B* in neuroblastoma cells was shown to cause the up-regulation of the let-7 members. Over-expression of *LIN28B* led to increased MYCN protein levels, which was explained by the fact that the let-7a was a direct post-transcriptional regulator of *MYCN* expression. Most importantly, this study demonstrated that *MYCN* was a down-stream target of LIN28 and silencing of *LIN28B* resulted in decreased cell viability and an increase in markers of cell differentiation. Transgenic mice expressing Lin28b in the neural crest developed tumors

characterized for the low expression of let-7, high MYCN levels, and a histology and location similar to human neuroblastomas. With these studies, *LIN28B* has emerged as a new oncogene in neuroblastoma and a novel therapeutic target.

6.3 Long non-coding RNAs

Compared with the extensive information available regarding miRNA, there is very little known about the expression or role of long non-coding RNAs (lncRNAs) in NB. This emerging area of research concerns RNAs of length >200nt, which lack protein-coding features such as open-reading frames, are not translated into proteins and exert their functional role as RNA transcripts.

One sub-group of lncRNAs whose expression has been investigated to some extent in neuroblastoma are the transcribed ultra-conserved regions (T-UCRs), transcripts from DNA segments that are at least 200bp in length and that are 100% conserved between human, rat and mouse genomes. Four hundred and eighty-one such genomic regions have been identified [176]. Calin *et al* carried out the first analysis of transcribed UCRs (T-UCRs) in cancer, demonstrating that approximately 9% of the 962 possible T-UCRs (sense + anti-sense) were aberrantly transcribed in either carcinomas or leukemias relative to normal tissue [177]. The authors further demonstrated the oncogenic potential of T-UC.73A by siRNA-mediated down-regulation, resulting in significantly increased apoptosis in a colorectal cancer cell line. Two neuroblastoma studies have demonstrated that analysis of UCR expression signatures can be applied to the evaluation of neuroblastoma tumors [178, 179]. Differential UCR expression profiles are associated with outcome in short-term versus long-term survivors with high-risk, stage 4 neuroblastoma [179]. In addition, Mestdagh *et al.*, found an expression signature of up-regulated T-UCRs in MNA compared to non-MNA tumors [178].

Genetic aberrations such as chromosomal gains and losses can contribute to the over- or under-expression, respectively, of transcripts encoded in these regions. A predominant unfavourable prognostic factor in neuroblastoma is the gain of chromosome arm 17q [3, 68]. The 2.3kb RNA ncRAN (non-coding RNA expressed in aggressive neuroblastoma) is one such transcript mapped to 17q, whose over-expression is present in neuroblastomas with partial gain of 17q, but interestingly not present in those with whole chromosome 17 gain [180]. The oncogenic potential of this transcript was shown by siRNA knockdown of ncRAN in SH-SY5Y neuroblastoma cells, and ectopic over-expression in NIH3T3 mouse fibroblast cells, resulting in significantly inhibited cell growth and an increase in anchorage dependent cell growth respectively [180].

Differentially Expressed in Neuroblastoma (DEIN) is a non-coding transcript (mapping to 4q33-34) whose expression shows no significant association with the common neuroblastoma aberrations of *MYCN* amplification, 1p/3p/11q deletion or 17q gain. However, it is highly expressed in stage 4S tumors compared with localized stage 1, 3 and 4 tumors and expression is significantly associated with event-free survival, although not an independent prognostic marker [181].

The lack of information regarding the identification of new lncRNAs and their putative functions is due in part to the fact that they do not exhibit the same level of conservation as protein-coding genes. Consequently it is not possible to assign possible function through sequence similarity, as can be done with some protein-coding

genes. In addition, determining that a transcript does not code for a protein is a complex process. Moreover, studies indicate that certain transcripts can function at both RNA and protein level [182, 183]. However, with the evidence amassed so far, and the identification of functional roles for certain lncRNAs in the regulation of processes such as DNA methylation or apoptosis [177, 184, 185], it is clear that these RNAs can play important roles in both normal and pathological physiology and represent a challenging new area of research in both normal development and disease.

7 Conclusions

Since the early 1980's we have progressed significantly in our ability to diagnose, stratify and treat neuroblastoma patients. Risk classification continues to be optimized, and clearly future approaches will need to integrate profiling of mRNA and microRNA expression, epigenetic modifications, and whole genome copy number variations with the current INRG system. This will require advances in technology which will allow us to screen patients in a time-effective, cost efficient manner. In parallel, novel therapeutics are being developed to target key regulators of the neuroblastoma genome and more refined treatment regimens are being designed based on our increasing knowledge of the pathogenesis of the disease. This progress is due greatly to our increased understanding of the fundamental genetic alterations associated with tumor behavior and patient outcomes.

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