N-myc and Noncoding RNAs in Neuroblastoma

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Abstract

Neuroblastoma is a pediatric tumor of the sympathetic nervous system. Amplification and overexpression of the MYCN proto-oncogene occurs in approximately 20% of neuroblastomas and is associated with advanced stage disease, rapid tumor progression, and poor prognosis. MYCN encodes the transcriptional regulator N-myc, which has been shown to both up- and downregulate many target genes involved in cell cycle, DNA damage, differentiation, and apoptosis in neuroblastoma. During the last years, it has become clear that N-myc also modulates the expression of several classes of noncoding RNAs, in particular microRNAs. MicroRNAs are the most widely studied noncoding RNA molecules in neuroblastoma. They function as negative regulators of gene expression at the posttranscriptional level in diverse cellular processes. Aberrant regulation of miRNA expression has been implicated in the pathogenesis of neuroblastoma. While the N-myc protein is established as an important regulator of several miRNAs involved in neuroblastoma tumorigenesis, tumor suppressor miRNAs have also been documented to repress MYCN expression and inhibit cell proliferation of MYCN-amplified neuroblastoma cells. It is now becoming increasingly evident that N-myc also regulates the expression of long noncoding RNAs such as T-UCRs and ncRAN. This review summarizes the current knowledge about the interplay between N-myc and noncoding RNAs in neuroblastoma and how this contributes to neuroblastoma tumorigenesis.

Introduction

Neuroblastoma is a malignant embryonic childhood tumor arising from primitive cells of the neural crest. It accounts for more than 7% of childhood malignancies and around 15% of cancer-related deaths in childhood (1). The human proto-oncogene MYCN is amplified in about 20% of neuroblastoma tumors (2). MYCN amplification is closely related to poor survival of the patients, despite all modern multimodal treatment efforts. In contrast, MYCN nonamplified, low-stage neuroblastoma, and tumors in infants, even when metastasized, have the propensity to differentiate into benign subtypes, or regress spontaneously. Because of its profound effect on clinical outcome, MYCN amplification is routinely used as a biomarker for treatment stratification.

Besides MYCN amplification, hemizygous loss of large segments on chromosome 1p defines another major genetic subtype of high-risk neuroblastoma. MYCN amplification and 11q are inversely correlated and can be found in about 70% of all metastatic tumors. Typically, both genetic subtypes occur with additional genetic alteration. Loss of chromosome 1p is frequently found in MYCN-amplified tumors, whereas 11q loss is significantly associated with gain of 7q and 3p, and 4p loss (3). Gain of 17q material is frequent in both 11q- and MYCN-amplified tumors, most often caused by unbalanced t(11q;17q) and t(1p;17q) translocations, respectively (2).

The transcription factor N-myc, which is encoded by MYCN on chromosome 2p24, belongs to the Myc family of DNA binding basic region/helix-loop-helix/leucine zipper (bHLHZip) proteins, in which c-Myc, L-Myc, and N-myc are the best characterized members (4). The genomic sequences of MYCN and c-MYC share wide structural homology. Both genes consist of 3 exons, where the first exon is untranslated and exons 2 and 3 encode the translated regions (5). N-myc and c-MYC proteins are of similar sizes (464 and 454 amino acids, respectively). However, the MYCN mRNA is longer, mainly because of a larger 3′-untranslated region (3′UTR). In addition to structural and sequence homologies within the Myc family, the function and biochemical properties of these proteins are closely related. Myc-proteins heterodimerize with the bHLHZip-protein Max to a transcription factor complex that binds to specific E-box DNA motifs (5′-CAGNGT-3′) and activates transcription of genes involved in diverse cellular functions, including cell growth and proliferation, metabolism, apoptosis and differentiation (6–9). N-myc preferentially binds to the E-box motifs CATGTG and CAACTG. Under MYCN-amplified conditions, however, N-myc becomes less specific and binds additionally to CATTGG and CATCTG.
(10). In addition to Myc, Max also dimerizes with the bHLHZip-proteins Mad/Mnt. These complexes also bind to E-box elements, but repress transcription through the recruitment of corepressors (11). Through interaction with Sp1 and Miz-1 at promoters, N-myc has been shown to silence gene expression by recruitment of the histone deacetylase HDAC1 (12, 13).

While c-MYC is expressed during all developmental stages and in a distinct pattern throughout the cell cycle of dividing cells (14, 15), N-myc expression is restricted mainly to the nervous system and mesenchymal tissues during particular embryonal stages (16).

Several individual N-myc downstream targets have been identified, including p53 (17), TERT (18), ODC1 (19), MCM7 (20), and MDM2 (21). The current knowledge and models on how these and other protein-coding N-myc targets upon deregulation may contribute to neuroblastoma formation have recently been reviewed by others (6, 9).

During the last 20 years, it has become increasingly apparent that the expressed non–protein-coding portion of the genome, the noncoding RNA (ncRNA), plays important infrastructural and regulatory roles in many cellular processes. The regulatory ncRNAs are commonly grouped into 2 classes based on practical reasons related to RNA purification protocols; small ncRNAs (i.e., miRNAs, siRNAs, piRNAs, etc. <200 nt) and a diverse group of long ncRNAs (lncRNA >200 nt). Dysregulation of ncRNAs has been found to have relevance in several diseases, including cancer, neurologic, cardiovascular, developmental, and other disorders (22, 23).

MicroRNAs (or miRNAs) are the most widely studied ncRNA and constitute an abundant class of endogenous small ncRNAs that negatively regulate protein expression in cells (24). The biogenesis and action of miRNAs have been comprehensively reviewed elsewhere (25, 26). The first miRNA, lin-4, was discovered in 1993 in the nematode Caenorhabditis elegans (27, 28). Since that time, miRNAs have been found in nearly every organism, from plants and simple multicular organisms to flies, vertebrates, and humans. MiRNAs are annotated and catalogued in the public-accessible database miRBase (www.mirbase.org; refs. 29–33), which was founded at the Sanger Institute in England and is now managed by the University of Manchester. The current miRBase release 19 (August 2012) annotates 25,141 mature miRNAs in 193 species, including 2042 unique mature human miRNAs.

Expression changes of even single miRNAs have profound effects on the protein composition in cells (34, 35). The degree of complementarity between the mature miRNA sequence and the target mRNAs determines the mechanism responsible for blocking gene expression. Near-perfect pairing, as commonly seen in plants, causes mRNA destruction through Ago-catalyzed mRNA cleavage (36, 37). In vertebrates, miRNA-mRNA interactions are most often through imperfect base pairing (24). Here, the precise mechanisms behind miRNA-mediated gene silencing are still scientifically debated. A current model argues that the process begins with initiation-targeted translational repression followed by a general mRNA destabilization scenario ultimately leading to mRNA decay (38).

As miRNAs tend to target many different mRNAs, and each mRNA may contain several to hundreds of different miRNA binding sites, it is obvious that the miRNA-mRNA regulatory network is extremely complex. It has been estimated that 30% to 60% of all human genes are regulated by miRNAs (39, 40); others suggest that small RNAs, including miRNAs, will have the potential to regulate all human genes (41). Established roles for miRNAs are their involvement in the development of organisms and organs, in cellular processes such as proliferation, differentiation, signal transduction, and apoptosis, in cell fate decisions and immunologic defense of viral attacks (reviewed in refs. 42 and 43). As a consequence of this broad function, miRNA biogenesis has to be tightly controlled. Deregulated miRNA expression has been associated with a diversity of diseases, including cancer; a fact attributed in the term “oncomirs” for cancer-related miRNAs. MiRNA transcription is regulated by several transcription factors, including oncogenes such as c-MYC (44, 45) and MYCN, and tumor suppressor genes such as TP53 (46).

Studying N-myc and miRNA Expression—General Aspects

Basically, 3 different approaches have been used to study the role of N-myc on miRNA expression in neuroblastoma: (i) comparison of miRNA expression profiles before and after experimental MYCN-knockdown or N-myc overexpression in MYCN-amplified or nonamplified neuroblastoma cell lines, respectively; (ii) comparison of miRNA expression profiles in MYCN-amplified and nonamplified primary tumors; and (iii) analysis of direct N-myc binding to miRNA promoters or promoters of the host genes, for example, by chromatin immunoprecipitation (ChIP).

The 2 very first studies investigating the role of N-myc on miRNA expression in neuroblastoma tumors were published by Chen and Stallings (47) and Schulte and colleagues (48) almost 5 years ago. Both studies profiled the miRNA expression in a smaller set of primary tumors (18 and 24 tumors including 6 and 7 with MYCN amplification, respectively) to define differentially expressed miRNAs between the MYCN-amplified and nonamplified groups. Using miRNA-specific real-time RT-PCR, Chen and Stallings profiled 157 known miRNAs, whereas Schulte and colleagues used a microarray approach, supplemented by real-time RT-PCR validation, to profile 384 miRNAs in both neuroblastoma tumors and cell lines. Both studies found subsets of miRNAs that were differentially expressed between MYCN-amplified and nonamplified tumors, indicating for the first time that miRNAs play a role in neuroblastoma pathogenesis.

In the following years, a rapidly growing number of subsequent studies extended and refined the knowledge on N-myc–regulated miRNAs, taking general methodologic considerations into account:
Tumor sample size

The heterogeneous genetic background of neuroblastoma tumors requires large tumor sets to delineate miRNA expression signatures for complex genetic subgroups. In one of the largest miRNA profiling studies in neuroblastoma so far, Bray and colleagues (49) profiled 430 miRNAs in a total of 145 primary neuroblastoma tumors, including 36 with MYCN amplification. They found both up- and downregulated miRNAs (14 and 23, respectively) when MYCN-amplified tumors were compared with nonamplified tumors. Importantly, they also determined large-scale genomic gains and losses in each tumor by array-comparative genomic hybridization and correlated the genomic localization of differentially expressed miRNAs to chromosomal gains and losses. About 15% of all detectable miRNAs changed expression as a result of chromosomal imbalances in the tumors, highlighting that gains or losses of miRNA encoding regions contribute significantly to miRNA deregulation in neuroblastoma, in addition to N-myc overexpression.

MYCN expression

Experimental systems using MYCN induction or knockdown do not reflect 2 sides of the same coin, but initiate 2 distinct biologic processes, where the former results in cell-cycle progression and proliferation, whereas the latter in differentiation and apoptosis.

Profiling platforms

The nature of miRNAs (small size and base-paired structure) poses a challenge for miRNA-detection techniques. Different technical platforms (e.g., northern blotting, high-throughput RT PCR techniques, microarray analyses, next-generation sequencing) may, therefore, generate partially diverging expression profiles, mandating confirmation between the platforms. Moreover, different normalization methods for miRNA expression data can affect the calculation of expression changes, which can result in a nonuniform interpretation of differentially expressed miRNAs (50).

Number of miRNAs

The number of investigated individual miRNAs varies between studies, especially over time, not least because the overall number of identified miRNAs (and other small RNA molecules) in the human genome is still increasing. Profiling studies based on ultradeep next-generation sequencing of the total small RNA transcriptome in neuroblastoma (51) have the potential to provide ultra-specific and absolute miRNA expression data in future studies.

Functional confirmation

Differential miRNA expression data should be supported by functional studies in vitro and in vivo to prove biologic relevance of each individual miRNA.

In the following sections of the review, functional studies on N-myc-regulated miRNAs will be summarized in detail.

N-myc Induces miRNA Expression

The mir-17-92 cluster

The mir-17-92 cluster, which is transcribed as a polycistrionic unit from chromosome 13, comprises 7 individual miRNAs (mir-17, mir-18a, mir-19a, mir-19b-1, mir-20a, and mir-20b-1; ref. 52). The transcription of mir-17-92 is directly activated by both c-MYC (53) and N-myc (48) oncogenes.

Fontana and colleagues (54) published the first comprehensive functional study on the MYCN-regulated mir-17-92 cluster in neuroblastoma. They confirmed the observation made by Schulte and colleagues (48), showing that miRNAs of the mir-17-92 cluster are higher expressed in tumors and neuroblastoma cell lines with high N-myc expression. By the use of ChIP, they validated direct binding of N-myc to several E-box motifs in the mir-17-92 promoter and showed transcriptional activation in luciferase reporter gene assays. Moreover, Fontana and colleagues shed light on the functional consequences of mir-17-92 overexpression in MYCN-amplified neuroblastoma cells: the tumor suppressor p21 (CDKN1A) was shown to be targeted by mir-17, and overexpression of mir-17 in nonamplified cells increased proliferation, colony formation and in vivo tumor growth. Vice versa, inhibition of mir-17 by antagonirs in MYCN-amplified cells decreased proliferation and tumorigenesis, and increased p21 expression. Surprisingly, antagonir-17 increased apoptosis in neuroblastoma cells; an effect not attributable to increased p21. Instead, mir-17 was found to additionally target BIM (BCL2 interacting mediator of cell death, or BCL2L11), a pro-apoptotic BH3-only member of the BCL2 (B-cell lymphoma 2) family. In conclusion, Fontana and colleagues proposed that mir-17 functions as a major effector of MYCN-mediated tumorigenesis, by targeting p21, whereas at the same time protecting MYCN-amplified cells from N-myc induced apoptosis through translational inhibition of BIM.

Other studies have confirmed direct binding of N-myc to the mir-17-92 promoter (50, 55), as well as a positive correlation between expression of MYCN and members of the mir-17-92 cluster in primary tumors and/or neuroblastoma cell lines (49, 51, 55–61). As miRNAs simultaneously target a variety of different mRNAs, it became clear that activation of the mir-17-92 cluster enables N-myc to turn multiple cellular processes toward malignant transformation. Beveridge and colleagues (62) showed that mir-17 and mir-20a target 3 differentiation-associated genes in neuroblastoma cells; BCL2, MEF2D (myocyte enhancer factor-2D) and MAP3K12. Another differentiation-associated protein, the estrogen receptor-α (ER-alpha), was also reported to be a target for miRNAs of the mir-17-92 cluster. ER-alpha is expressed in fetal sympathetic ganglia during human neuronal development and has been shown to be inversely correlated to MYCN expression in neuroblastoma tumors (55). Loven and colleagues showed that mir-18a and 19a target ER-alpha, providing a mechanism on how N-myc regulates ER-alpha expression. Stable knockdown of mir-18a inhibited proliferation and induced differentiation of MYCN-amplified neuroblastoma cells. Notably, Loven...
and colleagues showed that N-myc also binds to E-boxes of the mir-17-92 paralogous miRNA clusters mir-106b-25 (chromosome 7) and mir-106a-363 (chromosome X), enabling a concerted action of N-myc-activated miRNAs to synergize N-myc functions. In a genome-wide proteome analysis, Mestdagh and colleagues (63) used a tetracycline-inducible mir-17-92 expression system in nonamplified neuroblastoma cells (SHEP-TRmir-17-92) to show that 144 proteins were downregulated upon mir-17-92 induction, including multiple key effectors along the TGF-β signaling cascade. Both TGFBR2 and Smad2/Smad4 were shown to be direct targets of mir-17/20 and mir-18a, respectively (63). Interestingly, TGF-β responsive genes include CDKN1A and BCL2L11 in gastric cancer (64), both direct targets of mir-17-92 in neuroblastoma (54).

Recently, our research group reported that mir-92a is positively correlated to MYCN expression both in neuroblastoma cell lines and tumors. Mir-92a was further shown to directly target the tumor suppressor DKK3 (Dickkopf-3) mRNA, resulting in decreased secretion of DKK3 from neuroblastoma cell (65). This observation has been confirmed by De Brouwer and colleagues (66).

These studies illustrate how N-myc is able to regulate multiple steps of oncogenic processes through the activation of the mir-17-92 cluster (Fig. 1).

Mir-9

Another functionally characterized miRNA positively correlated to MYCN expression is mir-9. This miRNA is highly expressed in the brain and other neural tissues and coordinates the proliferation and migration of human neural progenitor cells (67). Ma and colleagues (57) used an inducible MYCN expression system and genome-wide ChIP-on-chip analyses to confirm that mir-9 (at the mir-9-3 locus) is directly activated by N-myc and that mir-9 targets the tumor suppressor E-cadherin (CDH1). E-cadherin is an ubiquitously expressed transmembrane glycoprotein on the surface of epithelial cells, with a pivotal role for cell–cell adhesion of adjacent cells. E-cadherin function is frequently lost in epithelial cancers and associated with invasion and metastasis. In neural crest development, during the process of neurulation, downregulation of E-cadherin allows the neural crest cell to detach from the neural tube and migrate along the migratory pathway (68). Ma and colleagues found that mir-9 was significantly higher expressed in 23 metastasized neuroblastoma tumors (stage IV, all MYCN-amplified), compared with 22 nonamplified tumors without metastases. They showed that mir-9 promotes motility and invasiveness of both human mammary epithelial and breast carcinoma cell lines through the suppression of E-cadherin. Moreover, the decrease in E-cadherin increased expression of the proangiogenic factor VEGFA through activated β-catenin signaling in the cells. The study by Ma and colleagues propose for the first time a model on how N-myc might be able to contribute to metastasis formation through the activation of a single microRNA (Fig. 2).

Mir-421

A link between disturbed double-strand break (DSB)-induced DNA damage response and an N-myc-activated miRNA has been reported by Hu and colleagues (69). The authors reported increased expression of mir-421 covarying...
In conclusion, this study reported a new mechanism for ATM dysregulation related to neuroblastoma tumorigenesis.

**N-myc is Predominantly a Repressor of miRNA Expression**

Although the expression of several miRNAs have been documented to correlate with *MYCN* expression, there is now growing evidence that N-myc predominantly acts repressive on the overall miRNA composition in *MYCN*-amplified neuroblastoma cells (47, 49, 59–61, 71) and upon N-myc induction in nonamplified neuroblastoma cells (55). Lin and colleagues (71) used real-time RT-PCR to profile the expression of 162 miRNAs in 66 primary neuroblastoma tumors (including 13 with *MYCN*-amplification) and found a nearly global downregulation of miRNAs in high-risk tumors, especially in those with *MYCN* amplification. The authors hypothesized that dysregulation in *Dicer* and/or *Drosha*, key enzymes in the miRNA-processing pathway, may contribute to the widespread miRNA downregulation. Indeed, both *Dicer* and *Drosha* were lower expressed in stage 4 tumors compared with other stages, with the most strikingly differential expression between stage IV and stage IVS. This suggests that repression of miRNAs may be involved in tumor progression.

**N-myc–Regulated Tumor Suppressor miRNAs in Neuroblastoma**

In the study by Chen and Stallings (47), *mir-184* was significantly downregulated in *MYCN*-amplified tumors and upregulated upon siRNA-mediated *MYCN*-knockdown in a *MYCN*-amplified neuroblastoma cell line. Overexpression of *mir-184* reduced cell viability of both *MYCN*-amplified and nonamplified cell lines through the induction of apoptosis and G1 cell-cycle arrest. A follow-up study by Foley and colleagues (72) confirmed the inverse correlation between N-myc and *mir-184* expression in primary tumors and showed that inhibition of *mir-184* by antagonir treatment increased proliferation of neuroblastoma cells. Moreover, they showed that *mir-184* directly targets AKT2 (protein kinase B beta). AKT2 is a downstream effector of the phosphatidylinositol 3-kinase (PI3K) pathway, one of the most potent prosurvival pathways in cancer. Activation of AKT is associated with poor prognosis in neuroblastoma (73). Finally, Tivnan and colleagues (74) used an *in vivo* murine xenograft model where *mir-184*-transfected *MYCN*-amplified or *MYCN* nonamplified neuroblastoma cells were orthotopically injected into CB-17/SCID mice. Tumors arising from *mir-184*-transfected cells were smaller than the controls, and mice survived longer. In summary, these comprehensive studies clearly established *mir-184* as a tumor suppressor in neuroblastoma.

Another tumor suppressor miRNA repressed by N-myc is *mir-542-5p*. Several studies have shown an inverse correlation between *mir-542-5p* and *MYCN* amplification in primary tumors (49, 51, 60, 75). In a large-scale profiling study of 430 miRNAs in 69 primary tumors, Schulte and colleagues (60) found increased expression of 4 miRNAs in...
MYCN-amplified tumors, whereas 35 miRNAs were repressed, including mir-542-5p. Mir-542-5p expression was found to be predictive for outcome, with a significantly higher expression in patients with event-free survival compared with relapsed patients. Bray and colleagues (75) profiled the expression of 449 miRNAs in 145 neuroblastoma tumors and correlated mir-542-5p expression to clinical data. Expression of mir-542-5p was nonrandomly distributed among tumor genetic subtypes, with lowest expression in MYCN-amplified tumors (77% completely lacking expression) and highest expression in stage 1,2,3 and 4S tumors (24% lacking mir-542-5p). Patients with tumors lacking mir-542-5p expression had the poorest prognosis independently of the MYCN status in the tumors (60, 75). Bray and colleagues further showed that mir-542-5p overexpression in MYCN-amplified and nonamplified neuroblastoma cells reduced invasiveness in vitro, and restricted tumor growth and metastasis in vivo when cells were orthotopically injected into CB-17/SCID mice.

Genome and Small RNA Transcriptome – Wide Analyses of N-myc – Regulated miRNA Expression

Two studies have recently used deep sequencing approaches to analyze MYCN-regulated miRNA expression in neuroblastoma (51, 61). Schulte and colleagues (51) used the SOLiD v3 sequencing platform to compare the total small RNA transcriptome in 5 unfavorable MYCN-amplified tumors with 5 favorable non-amplified tumors. Analyzing the absolute number of miRNA reads, there was a trend toward a higher proportion of mature miRNAs in the favorable patient group, indicating a possible global suppression of miRNA transcription in MYCN-amplified tumors. Expression data of 204 miRNAs were validated by real-time RT-PCR with good correlation between the technical platforms. The SOLID sequencing data confirmed previously data on differential expression in MYCN-amplified versus non-amplified tumors, including the mir-17-92 cluster and mir-181 (positive N-myc-correlation) and mir-542-5p (nearly absent in MYCN-amplified tumors). In total, 76 miRNAs were differentially expressed between MYCN-amplified and non-amplified tumors (43 upregulated and 33 downregulated). This study also allowed the discovery of several new miRNAs in neuroblastoma and revealed valuable insight into miRNA editing and distribution of mir-5p/-3p and mir* forms. Cluster analysis was able to exactly separate the 2 clinical outcome groups based on their differential miRNA expression, indicating that the miRNA transcriptome reflects tumor aggressiveness (51).

Very recently, Shohet and colleagues (61) conducted a genome-wide search for N-myc binding sites in promoters driving miRNA expression in neuroblastoma. Using a combination of ChIP and the Illumina GA-I sequencing platform (ChIP-seq) in a neuroblastoma cell line with inducible N-myc expression, they identified 20 gene promoters, hosting a total of 30 miRNAs, to which N-myc specifically bound to E-box motifs. The majority of host genes that were correlated with survival were downregulated by high N-myc levels, suggesting a tumor suppressor function for these host genes as well as the coexpressed miRNAs. However, functional in vitro and in vivo studies of 2 MYCN-regulated intronic miRNAs (mir-591 and mir-558) identified tumor suppressor functions for mir-591 as expected, whereas mir-558 was reported to function as an oncomir.$^3$

These seemingly contrasting results probably highlight the complexity of oncogenes and downstream miRNAs in aggressive cancers. Indeed, it is well-established that deregulated MYC proteins on one hand exhibit oncogenic activities that contribute to cancer development, but on the other hand also activate antitumorigenic responses, for example, through activation of p53 (8).

C-MYC/N-myc – Induced miRNAs Repress Gene Networks

In a large-scale miRNA expression study, Mestdagh and colleagues (59) profiled the expression of 430 miRNAs in 95 neuroblastoma tumors and delineated a signature of 50 unique miRNAs differentially expressed between MYCN-amplified and MYCN single-copy tumors (16 upregulated and 34 downregulated miRNAs). Interestingly, the miRNA signature further delineated 2 distinct tumor subgroups within the MYCN single-copy group: tumors with high or low c-MYC expression. The 3 tumor groups defined by the 50-miRNA signatures correlated well with the clinical stage and prognosis. Mestdagh and colleagues concluded that MYCN/c-MYC signaling rather than MYCN amplification alone underlies the differential expression of miRNAs in neuroblastoma. To identify mRNA targets downstream of the MYCN/c-MYC–regulated miRNAs, they integrated mRNA and miRNA expression data sets from 40 neuroblastoma tumors and calculated correlations between each of the 50 miRNAs and around 15,000 mRNAs. In the group of miRNAs with inverse miRNA correlation, significant 3′UTR seed enrichment was only found for the 16 N-myc–activated miRNAs, indicating that these miRNAs have a widespread effect on differential gene expression in high-risk neuroblastoma. One-third of the miRNAs were predicted targets of 2 or more MYCN/c-MYC–activated miRNAs, indicating a concerted action toward target gene suppression. Low expression of predicted mRNA targets in the tumors correlated with a particular poor patient prognosis. MYCN/c-MYC–activated miRNAs were predicted to repress several pathways known to be involved in neuroblastoma, including integrin signaling. In summary, the study by Mestdagh and colleagues comprehensively showed widespread transcriptional repression of coding genes by MYCN/c-MYC through miRNA induction, serving as an additional mechanism of MYCN/c-MYC–induced oncogenicity.

MYCN-Driven miRNA Expression in a Murine Transgenic Neuroblastoma Model

The TH-MYC mouse is a widely used transgenic neuroblastoma model in which tumorigenesis is driven by neural crest-specific expression of the human MYCN transgene.
N-myc Alters Expression of Other Noncoding RNAs

Long noncoding RNAs (lncRNAs) belong to a diverse group of regulatory ncRNAs. LncRNAs are commonly defined as 200–100,000 nt long mRNA-like transcripts lacking significant open reading frames (79). The functions of lncRNAs are far from understood, but it is clear that this poorly conserved group of ncRNAs is involved in diverse mechanisms for widespread regulation of chromatin modifications and gene expression (80).

Compared with the 2042 known unique mature human miRNAs (miRBase v19), the number of lncRNAs has been estimated to be at least as numerous as miRNAs (81). LncRNAs have been shown to be aberrantly expressed in several human diseases, including cancers (79, 82).

Transcribed ultraconserved regions (T-UCR) are 200–779 bp lncRNAs expressed from 481 genomic regions that are 100% conserved between human, rat, and mouse (83). T-UCRs are widely expressed in neuroblastoma, and a signature based on the expression of 28 T-UCRs has been shown to be associated with good outcome in noninfect patients with metastatic disease (84). Furthermore, Mestdag and colleagues (85) investigated the global T-UCR expression in neuroblastoma and found a signature of 7 T-UCRs significantly upregulated in MYCN-amplified tumors (n = 18) compared with nonamplified tumors (n = 31). Of these, 7 (uc.350, uc.379, and uc.460) were also upregulated when N-myc expression was induced in the SHEP-MYCN-ER cell line, supporting that these T-UCRs are N-myc responsive. These data indicate that differential T-UCR expression correlates with clinicogenetic parameters such as MYCN amplification (85).

The most frequent genetic abnormality in neuroblastomas is chromosome 17q gain. Gain of 17q has been shown to be linked to advanced stage disease and amplification of the MYCN oncogene (86, 87). Several candidate genes of “17q gain” have been proposed, including a lncRNA called ncRAN (noncoding RNA expressed in aggressive neuroblastomas). ncRAN consists of 2 transcripts, Nbla10727 (2186 nt) and Nbla12061 (2087 nt), which are splice variants of the same gene mapped to chromosome region 17q25.1. Both transcripts are upregulated in advanced neuroblastomas with 17q gain. High or moderate levels of ncRAN expression were also observed in neuroblastoma cell lines with MYCN amplification, most of which had 17q gain. Furthermore, suppression of ncRAN expression significantly inhibited cell growth in a neuroblastoma cell line and overexpression of ncRAN in mouse fibroblasts enhanced anchorage-independent growth (88). Recently, expression of ncRAN was found to be upregulated in bladder cancers compared with normal tissue. Overexpression of ncRAN in a superficial low-ncRAN expressing bladder cancer cell line enhanced cell proliferation and invasion, whereas shRNA knockdown of ncRAN in a high-ncRAN expressing invasive bladder cancer cell line sensitized the cells to chemotherapeutic drugs (89). According to these data, ncRAN appears to have oncogenic properties in several cancers.

Despite its relative small size (~130 nt), NDM29 (Neuroblastoma Differentiation Marker 29) is classified as a lncRNA. This Alu-like RNA is transcribed by RNA polymerase III from the first intron of the ASCL3 (Achaete-Scute-Like homologue 3) gene. Stable overexpression of NDM29 in a MYCN-amplified neuroblastoma cell line induced neuronal differentiation, reduced the proliferation rate, reduced c-kit expression, induced anchorag-dependent growth, and decreased anticancer drug resistance through MDR1 (multidrug resistance 1) downregulation (90). Although the status of N-myc expression was not reported in these experiments, it is interesting to notice that NDM29 overexpression coincides the effects of MYCN knockdown in MYCN-amplified neuroblastoma cells (91–93).

DEIN (differentially expressed in neuroblastoma) is a putative lncRNA expressed as 5 isoforms. Isoform A (4186 bp) and isoform B (5278 bp) are the 2 major transcript variants of DEIN in primary neuroblastomas. DEIN has been shown to be differentially expressed in neuroblastoma subtypes, with higher expression in tumors with good prognosis and spontaneous regression (children <1 year and in Stage IVS). No significant association between DEIN and MYCN amplification was reported (94). The expression of DEIN has been shown to be tightly linked to expression of the PHOX2B-downstream target HAND2 through a
bidirectional promoter. This suggests a role for DEIN in suppression of cell proliferation and promotion of differentiation (95).

N-myc Expression is Regulated by miRNAs

Finally, the interaction between N-myc and miRNAs is mutual, as MYCN itself is targeted by miRNAs. The functionally best-characterized MYCN-targeting miRNA is mir-34a (96, 97), which is located at chromosome 1p36, a region frequently deleted in MYCN-amplified neuroblastoma tumors (1). Overexpression of mir-34a in MYCN-amplified neuroblastoma cell lines decreased N-myc levels, inhibited proliferation, and induced apoptosis. Interestingly, mir-34a is transcriptionally activated by p53 (46), implicating that deletion of mir-34a has similar cellular consequences as p53 deficiency (97). In addition to MYCN, mir-34a targets BCL2 (96) and E2F3 (98), making mir-34a a multifaceted tumor suppressor miRNA in neuroblastoma. Recently, our research group systematically investigated the MYCN 3’UTR for miRNA binding sites (99). In addition to mir-34a, we validated mir-34c, mir-449, mir-19, mir-29, mir-101, and let-7/mir-202 as MYCN-targeting miRNAs (Fig. 3). Overexpression of mir-101 and let-7e in MYCN-amplified neuroblastoma cells diminished N-myc levels, and reduced proliferation and clonogenic growth. While mir-101 is generally sparsely expressed in neuroblastoma and has established tumor-suppressor functions in other cancers ([100] and references therein), let-7 family members are upregulated during neuroblastoma cell differentiation (47, 56, 78, 101–103), suggesting tumor suppressor functions for these miRNAs in neuroblastoma.

Summary

The proto-oncogenic transcription factor N-myc is emerging as an important regulator of non–protein-coding gene expression in neuroblastoma. Most studies have focused on the interplay between MYCN expression and a group of small regulatory noncoding RNAs, the microRNAs (miRNAs). From several studies published during the last 5 years, it is evident that N-myc both activates and represses the expression of several miRNAs (summarized in Supplementary Tables S1 and S2), many of them known to have important roles in cancer progression. MiRNA induction by N-myc (and also c-MYC) is associated with a widespread repression of coding genes and disease-relevant pathways in neuroblastoma cells, suggesting that miRNA-controlled regulation of certain groups of mRNAs may function as an additional mechanism of MYCN/c-MYC-induced oncogenicity. Indeed, among the targets of MYCN-responsive miRNAs (mir-17-92 cluster, mir-9 and mir-421) are mRNAs of tumor suppressors, and genes involved in the metastatic process. On the other hand, miRNAs with tumor suppressor functions (mir-184 and mir-542-5p) have been shown to be inversely correlated to MYCN expression. In fact, miRNA repression seems to be the predominant consequence of MYCN activation, as high MYCN expression is identified with a global repression of miRNAs, possibly through impaired Drosha/Dicer expression. Vice versa, some mRNAs (mir-34a, mir-101, and let-7) have been documented to target the MYCN mRNA, resulting in reduced N-myc expression and decreased proliferation of MYCN-amplified neuroblastoma cells.

Long noncoding RNAs (lncRNAs) have recently been implicated in playing important regulatory functions in several diseases, including cancers. It is now becoming clear that N-myc levels also affect the expression of lncRNAs (such as T-UCRs and ncRAN) in neuroblastoma. Understanding the mechanisms by which N-myc alters the expression of the diverse class of regulatory lncRNAs, and through which targets and pathways deregulated lncRNAs may subsequently contribute to oncogenic transformation in aggressive neuroblastoma, will possibly explain missing molecular links in the pathogenesis of this disease, and further elucidate how oncogenic N-myc signaling is executed. A distinct role for noncoding RNAs in neuroblastoma is further supported by the fact that several noncoding RNAs with functional roles were initially identified because of their genomic location in areas of frequent losses or gains in advanced disease stages.

In conclusion, further understanding of the effect of N-myc on the expression of non–protein-coding genes in the genome will help to understand the pathogenesis of aggressive neuroblastomas, and provides a basis for novel targeted therapies for neuroblastoma treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Buechner, C. Einvik
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Buechner, C. Einvik

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References


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