Published in final edited form as:
Curr Pharm Des. 2009 ; 15(4): 456–462.

MicroRNA Involvement in the Pathogenesis of Neuroblastoma: Potential for MicroRNA Mediated Therapeutics

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Abstract

Neuroblastoma arises from precursor cells of the sympathetic nervous system and presently accounts for 15% of all childhood cancer deaths. These tumors display remarkable heterogeneity in clinical behavior, ranging from spontaneous regression to rapid progression and resistance to therapy. The clinical behavior of these tumors is associated with many factors, including patient age, histopathology and genetic abnormalities such as *MYCN* amplification. More recently, the dysregulation of some miRNAs, including the miR-17-5p-92 cluster and miR-34a, has been implicated in the pathobiology of neuroblastoma. MiR-17-5p-92 family members act in an oncogenic manner while miR-34a has tumor suppressor functions. The evidence for the contribution of miRNAs in the aggressive neuroblastoma phenotype is reviewed in this article, along with exciting possibilities for miRNA mediated therapeutics.

Keywords

MicroRNA; Neuroblastoma; MYCN; Chromosomal Imbalance

Mature, biologically-active miRNAs are 21 to 22 nucleotides in length and are processed from much larger transcripts. MicroRNAs (miRNAs) regulate gene expression at a post-transcriptional level by targeting messenger RNAs (mRNAs) that have sequence similarity in their 3' untranslated region (UTR) with the miRNA. In animals, the sequence complementarity between a miRNA and a region of an mRNA's 3' UTR can lead to either degradation of the targeted mRNA, or in translational inhibition. The net result is a deceased amount of a particular protein. The effects of miRNAs can be remarkably complex, given that an individual miRNA can target multiple mRNAs, and individual mRNA's can be regulated by diverse miRNAs.

MiRNAs play major roles in the differentiation of neural and other cell types [1], and the dysregulation of these sequences clearly contributes to tumorigenesis in many forms of cancer. As detailed in many excellent review articles, numerous miRNAs are over-expressed in different forms of cancer, acting in a dominant oncogenic fashion, or under-expressed, behaving as recessively acting tumor suppressors [2-6]. The purpose of this article is to review the role of miRNA dysregulation in the pathogenesis of neuroblastoma, a pediatric cancer arising from precursor cells of the sympathetic nervous system [7], and to access the potential of miRNA mediated therapy for the treatment of these tumors.

Neuroblastoma tumors are particularly noted for extensive heterogeneity in clinical behavior, ranging from spontaneous regression to aggressive clinical course and death due to disease. The clinical behavior of these tumors is correlated, to some extent, with specific genomic abnormalities. Tumors in infants that are characterized primarily by hyperdiploidy and few structural chromosome abnormalities have a high propensity to spontaneously regress or

differentiate into benign ganglioneuroma. In contrast, tumors from children >1.5 years of age often become refractory to treatment and exhibit many recurrent chromosomal imbalances and/or high level amplification of the *MYCN* oncogene. Some of the recurrent abnormalities are nonrandomly distributed and form the basis for dividing neuroblastoma into genetic subtypes, as reviewed by Stallings [8].

Neuroblastoma tumors with amplification of the *MYCN* transcription factor and/or loss of distal chromosome 1p and gain of 17q represent a major genetic subtype of metastatic neuroblastoma with the worst prognosis [9-11]. Tumors with hemizygous loss of a large segment on chromosome 11q represent another major genetic subtype of metastatic neuroblastoma [10, 12]. These tumors are preferentially associated with loss of chromosome 3p [13-15] and have a suboptimal clinical outcome [14,16]. Like *MYCN* amplified tumors, 11q- tumors usually have gain of 17q. There are many additional recurring large-scale chromosome imbalances found in neuroblastomas, including loss of 4p, 9p, 14q, and gain of 1q, 7q, 2p, and 11p (see [17] for review). Large-scale chromosomal imbalances, such as these, have been shown to contribute to the dysregulation of miRNAs in various forms of cancer [18], and the possibility that they are important in dysregulating miRNAs in neuroblastoma is an open and interesting question.

Expression profiling of miRNAs in primary neuroblastoma tumors

Recently, Chen and Stallings [19] demonstrated that many miRNA loci are differentially expressed in different genomic subtypes of neuroblastoma and that these subtypes can be identified on the basis of miRNA expression patterns. In particular, tumors of the *MYCN* amplified (MNA) subtype could be quite accurately classified on the basis of miRNA expression profiling, as illustrated in Fig. (1). Classification of high stage 11q- versus low stage hyperdiploid tumors was less accurate. This is not surprising since 11q- and hyperdiploid tumors are also less accurately classified on the basis of mRNA expression profiling [20,21]. It was further noted that a large number of miRNAs appeared to be down-regulated in the MNA subtype relative to the 11q- or hyperdiploid subtypes, indicating that MYCN might mediate a tumorigenic effect, in part, by down-regulating miRNAs that have pro-apoptotic or pro-differentiation effects [19]. MiR-184 is an example of a miRNA that is down-regulated in the MNA subtype which has pro-apoptotic effects when ectopically up-regulated in neuroblastoma cell lines [19]. Interestingly, a related transcription factor, *MYC*, apparently directly down-regulates a number of miRNA loci in other forms of cancer [22].

In contrast to Chen and Stallings [19], Schulte et al [23] did not observe under-expression of miRNAs in their analysis of miRNA expression in neuroblastoma primary tumors. This could be do to the fact that the later group analyzed a smaller set of tumors which were classified only as MNA or non-MNA, whereas Chen and Stallings [19] classified tumors as being MNA, 11q- or hyperdiploid. Both groups identified a smaller number of miRNAs which were up-regulated in the MNA tumors which might be acting in an oncogenic fashion by promoting cell proliferation [19,23]. These results suggest that miRNAs play a role in neuroblastoma pathobiology and that the inclusion of miRNA expression profiling data could potentially improve the accuracy of prognostic information derived from gene expression signatures based on mRNA [24,25].

Endogenous over-expression of MiR-17-5p in neuroblastoma

Fontana et al [26] demonstrated that the five miRNAs mapping within the miR-17-5p-92 polycistronic cluster (miR-17-5p, -18a, -19a, -20a and -92) were expressed at higher levels in neuroblastoma cell lines exhibiting over-expression of *MYCN* relative to cell lines with low levels of this transcription factor. Using chromatin immunoprecipitation and luciferase reporter constructs, Fontana et al [26] further demonstrated that MYCN binds directly to several sites in both the 5' and 3' regions of the miR-17-5p-92 cluster, causing up-regulation of the

polycistron. Some of these miRNAs clearly act as oncogenes in other forms of cancer [27], so that they are of general importance in cancer biology.

Remarkably, ectopic over-expression of the miR-17-5p-92 cluster in the *MYCN* single copy neuroblastoma cell line SK-N-AS was shown to increase cell proliferation rates both *in vitro* and *in vivo* [26]. This group then focused their studies on miR-17-5p, which is predicted to target the p21 tumor suppressor gene (*CDKN1A*). They experimentally validated that this miRNA directly down-regulates p21 by binding to the 3' UTR, and that p21 down-modulation by either miR-17-5p or by siRNA is responsible for enhanced cell proliferation and tumorigenesis. MiR-17-5p also was shown to have anti-apoptotic effects by targeting the pro-apoptotic gene, *BIM*. To demonstrate a potential therapeutic application of their studies, Fontana et al [26] demonstrated that injection of a miR-17-5p antagomir into nude mice that had been injected with cells from the MNA LAN-5 neuroblastoma cell line significantly inhibited tumor growth *in vivo*. There was a significant down-modulation of miR-17-5p activity in these xenograft tumors and a reciprocal increase of both p21 and *BIM* protein levels. Notably, thirty percent of cases treated with the miR-17-5p antagomir showed complete regression of the tumor and a significant increase in apoptosis.

The pattern of MiR-17-5p expression in primary tumors, however, appears to be rather complex since high levels of miR-17-5p expression were detected in both MNA primary tumors as well as in nearly half of the non-MNA tumors that were analyzed [26]. The non-MNA tumors were from patients with different disease stages (stages 1 through 4). This result is consistent with the results of Chen et al., [19], who did not observe statistically significant differential expression of miR-17-5p in high stage MNA versus 11q- tumors or in low stage hyperdiploid tumors (this group observed over-expression of only miR-92 from the miR-17-5p-92 cluster in the MNA subgroup). The mechanism leading to different levels of mature miRNA for members of the same polycistronic cluster is uncertain. Clearly, analysis of larger tumor sets, with more refined genomic subgroupings, is required to fully understand the patterns of expression of the miR-17-5p-92 family. Nevertheless, the use of a miRNA antagomir to knock-down the expression of an endogenous miRNA *in vivo* shows great promise for microRNA mediated therapy of neuroblastoma.

MiR-34a induces apoptosis in neuroblastoma cell lines

In addition to miRNAs behaving in a dominant, oncogenic manner in neuroblastoma, there is also evidence that miRNAs can act in a recessive, tumor suppressive fashion. The first miRNA identified in neuroblastoma with tumor suppressor properties was miR-34a, which maps to the chromosomal region 1p36.23 that is commonly hemizygously deleted, particularly in tumors exhibiting *MYCN* amplification. Welch et al [28] initially demonstrated that this miRNA is expressed at lower levels in tumors with 1p deletion, and that artificial ectopic over-expression of miR-34a in a number of neuroblastoma cell lines leads to the arrest of cell proliferation and the induction of a caspase-mediated apoptotic pathway, as illustrated in Fig. (2). Welch et al [28] further demonstrated that the 3' UTR of the *E2F3* transcription factor, a potent inducer of cell cycle progression, was directly targeted by miR-34a. Given that E2F family members are potent inducers of cell cycle progression, and in certain cellular contexts have proapoptotic effects [29], the down-regulation of *E2F3* by miR-34a would appear to contribute to the anti-proliferative phenotypic effects of miR-34a up-regulation. The anti-proliferative effects of miR-34a in neuroblastoma cells have been confirmed by other groups [30,31].

Interestingly, *MYCN* is also a computationally predicted target of miR-34a. Although Welch et al [28] were unable to experimentally validate *MYCN* as a miR-34a target with their luciferase reporter constructs, Wei et al [31] definitively demonstrated that MYCN is a direct target of miR-34a [30,31]. In fact, there are two functional miR-34a target sites in the *MYCN* 3' UTR

[31]. The targeting of *MYCN* by miR-34a provides an explanation as to why loss of chromosome 1p occurs preferentially in MNA tumors, since a functionally active miR-34a locus would negate the effects of *MYCN* amplification by blocking the translation of the *MYCN* mRNA into MYCN protein. The direct down-regulation of MYCN by ectopic up-regulation of miR-34a would also be expected to significantly contribute to the anti-proliferative phenotype induced by this miRNA. In addition to targeting *E2F3* and *MYCN*, other genes involved with cell proliferation or apoptosis have been shown to be targeted by miR-34a, including *BCL2* [32], *CCND1* [33] and *CDK6* [33].

Extremely high resolution comparative genomic hybridization analysis of microarrays has indicated that virtually all of the deletions affecting chromosome 1p in neuroblastoma appear to be hemizygous deletions [34], so that it is likely that other genetic mechanisms are involved with lowering the expression of the non-deleted miR-34a allele. DNA sequence analysis of miR-34a failed to detect any mutations at this locus in 30 tumors [30], while epigenetic silencing of miR-34a through hypermethylation of the promoter region is another possibility that warrants further study. Interestingly, several groups have now demonstrated that the p53 transcription factor directly binds to the 5' region of the miR-34a gene and is required for the up-regulation of this miRNA [32,35-38]. Since MYCN directly up-regulates the expression of the *MDM2* gene, whose product antagonizes the action of p53, it seems plausible that MYCN over-expression is indirectly down-regulating the expression of miR-34a, as illustrated in Fig. (3). P53, which is mutated in approximately 50% of all human tumors, is rarely mutated in primary neuroblastomas [39], possibly because of the down-modulation of miR-34a, a down-stream mediator of p53 pathway. It will be interesting to determine if there is a significant inverse correlation between miR-34a expression and p53 mutations in other forms of cancer as well.

Intriguingly, initial results suggest that a miR-34 family member mapping to a region that is commonly deleted on chromosome 11q, miR-34c, also has anti-proliferative effects when artificially up-regulated in neuroblastoma cell lines [30]. Similar to miR-34a, miR-34c is also directly regulated by p53 [40]. Since loss of 11q represents a neuroblastoma subtype that is primarily independent of the MNA/1p- subtype, it is tempting to speculate that loss of at least one miR-34 family member is an essential property of most metastatic neuroblastoma. The possibility that miR-34 family members have complementary functions in the neuroblastoma cell of origin is an intriguing hypothesis requiring further testing. Clearly, the development of methods leading to the up-regulation of endogenous miR-34a or ectopic introduction of miR-34a in *MYCN* amplified neuroblastoma could potentially be of significant benefit in the treatment of neuroblastoma, as could the restoration of miR34c activity in 11q- tumors.

Altered Regulation of MiRNAs in Response to Retinoic Acid Treatment

The retinoid derivative, all-trans-retinoic acid (ATRA), can induce some neuroblastoma cell lines, such as SK-N-BE, to undergo differentiation or apoptosis [41]. A related compound, cis-retinoic acid, is currently used as part of the treatment regimen for some neuroblastoma cases [42], thus, a detailed understanding of the molecular pathways affected by retinoic acid should be of considerable interest. The down-regulation of *MYCN* occurs at a very early stage following exposure of neuroblastoma cells to retinoic acid [41], which would be expected to alter the transcript levels of many additional genes. Indeed, using mRNA expression microarray profiling, Truckenmiller et al [43] demonstrated that the expression of numerous genes are altered very early after exposure of neuroblastoma cells to retinoic acid and that these expressional alterations are consistent with neural cell differentiation in many respects. Given that MYCN has been shown to directly regulate some miRNAs, one might also expect to observe alterations in some of these sequences in response to the down-modulation of *MYCN* following ATRA treatment. That specific miRNAs undergo expressional alterations in

SK-N-BE cells induced to undergo differentiation in response to ATRA was shown to be the case by both Chen and Stallings [19] and by Laneve et al [44].

Laneve et al [44] analyzed the expression of 70 miRNAs that are known to be expressed in neuronal cells at different time points in SK-N-BE cells treated with ATRA. They identified 14 miRNAs that were up-regulated, and among these up-regulated miRNAs, the authors focused on miR-9, 125a and 125b for further study because these miRNAs were also up-regulated in other neuronal cell lines treated with ATRA. Ectopic over-expression of these miRNAs in SK-N-BE cells in the absence of ATRA, both individually and in different combinations, had significant anti-proliferative effects and could be correlated with a 60% reduction in MYCN levels. None of these miRNAs appear to directly target the *MYCN* 3' UTR, so the authors concluded that the down-modulation of *MYCN* must be a secondary effect. Notably, knock-down of these miRNAs using locked nucleic acid oligonucleotides prior to exposing the cells to ATRA somewhat negated the effects of this compound, resulting in higher levels of cell proliferation. These observations support the notion that the up-regulation of some miRNAs in response to ATRA treatment contribute to the process of neuroblastoma cell differentiation. Finally, it was shown that all three of these miRNAs target a truncated isoform of a neurotrophic tyrosine receptor kinase, *NTRK3* (also known as *TRKC)* and that siRNA mediated post-transcriptional knock-down of this isoform alone has antiproliferative effects that mimic the effects, to some extent, of the endogenous miRNA up-regulation induced by ATRA [44].

Chen et al [19] analyzed the expression of 32 miRNAs that were differentially expressed in favorable and unfavorable neuroblastoma tumor subtypes in ATRA treated SK-N-BE cells. Seventeen of these loci were up-regulated while four loci were down-regulated. Interestingly, one of the down-regulated loci was miR-92, part of the miR-17-5p-92 cluster that has been shown to be over-expressed in MNA tumors and directly up-regulated by MYCN [26]. Thus, down-regulation of this miRNA is entirely consistent with the down-regulation of *MYCN* that occurs very soon after ATRA exposure [41]. Six of the loci studied by Chen et al [41] overlapped the set of loci studied by Laneve [44]. Among these six loci, let-7a and 7b were shown to be up-regulated in both studies, while four loci showed expressional alteration in only one of the studies. These discrepancies could be related to the methods used to initially screen for expressional differences, one study having used Northern blots [44] while the other used QPCR [19].

Clearly, miRNAs play important roles in mediating the effects of ATRA, a compound used in the treatment of neuroblastoma and other malignancies. Further analysis to identify other miRNAs that might be involved with neuroblastoma cell differentiation is likely to produce further intriguing results with potential therapeutic applications. Indeed, it is quite possible that some of the most important miRNAs expressed in neuroblastoma are not even currently annotated in the Sanger miRNA Registry (http://microrna.sanger.ac.uk/sequences/). Afanasyeva et al [45] identified 14 novel miRNAs expressed in neuroblastoma following the cloning and sequencing of small RNAs from a variety of neuroblastoma tumors and cell lines. The incorporation of such miRNAs into PCR or microarray based expression analysis systems will be essential for obtaining a more complete understanding of the role of miRNAs in neuroblastoma pathogenesis.

The biology of microRNAs is incomplete

Our understanding of the role of miRNAs in the pathogenesis of neuroblastoma depends, to a large degree, on our understanding of the basic biology of miRNAs. A number of recent papers suggest that this understanding is far from complete. For example, let-7 family members have recently been shown to enhance translation of target mRNA sequences in cells undergoing cell

cycle arrest, while inhibiting translation in cells that are proliferating [46]. Many members of the let-7 family are computationally predicted to target *MYCN* (Pictar and Target scan algorithms), but paradoxically, my group [19] and others [23] have shown that let-7a and let-7b are up-regulated in MNA primary tumors. One would not expect them to be up-regulated in MNA tumors given that this would negate the effects of *MYCN* up-regulation. Is it possible that let-7 family members are enhancing the translation of *MYCN*? Alternatively, it is intriguing that some RNA binding proteins, such as DND1, can counteract the inhibitory effects of certain miRNAs by blocking access to their binding sites on the mRNA 3' UTR [47]. Thus, it is tempting to speculate that an RNA binding protein is protecting the 3' UTR of *MYCN* from miRNAs such as let-7a and b. A candidate RNA binding protein would be HUD, which is already known to bind to the *MYCN* 3'UTR and increase the stability of the mRNA [48]. It should be noted that let-7a has yet to be experimentally validated for targeting *MYC*N, but that it is known to target a related gene family member, *c-MYC* [49].

Conclusions

The prospects for miRNA mediated therapeutics in neuroblastoma look very promising, particularly in view of the fact that the injection of miR-17-5p antagomirs successfully reduced tumor growth in vivo, and was the first demonstration of the use of a miRNA antagomir for the control any type of cancer *in vivo* [26]. The role of miRNAs in neuroblastoma pathogenesis, however, appears to be very complex and linking different miRNAs to different genetic pathways is clearly still in its infancy. It is also important to realize that our understanding of the basic function of miRNAs is still in its early days. For example, recent studies have shown that certain RNA binding proteins can block the effects of miRNAs and that under certain conditions of cell cycle arrest; miRNAs can actually enhance translation of some mRNAs as opposed to inhibiting translation. Whether this phenomena plays a role in neuroblastoma remains to be discovered.

Acknowledgments

Funding from Science Foundation Ireland, the U.S. National Institutes of Health, Cancer Research Ireland, the Association of International Cancer Research and the Children's Medical and Research Foundation (Dublin) is gratefully acknowledged.

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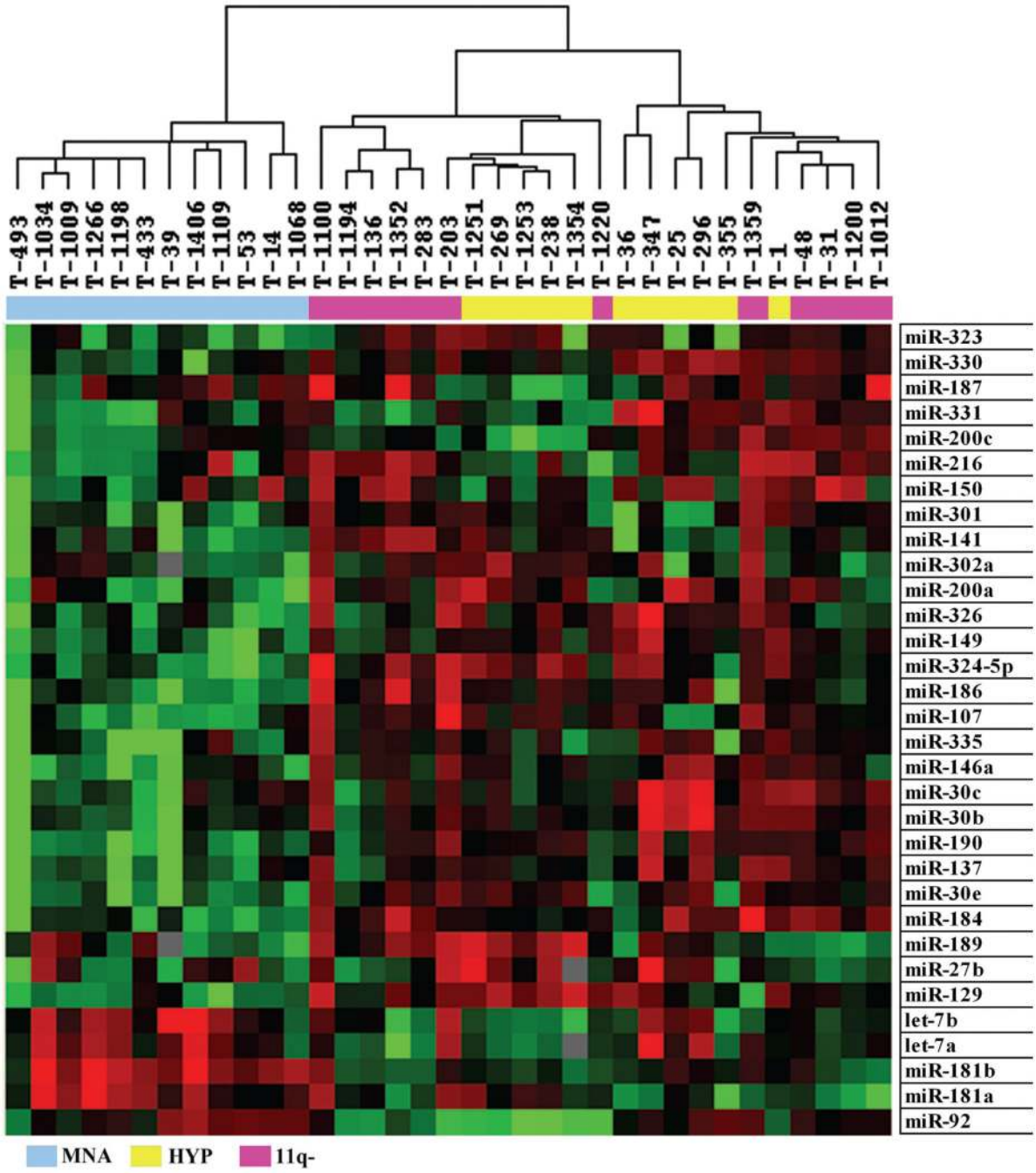

Fig. (1).

Heat map summarizing the patterns of expression for 32 miRNA loci that were differentially expressed in neuroblastoma tumor subtypes (low stage hyperdiploid tumors with favorable histopathology, yellow; high stage 11q-tumors with unfavorable histopathology, pink; and high stage *MYCN* amplified tumors with unfavorable histopathology, blue). High expression is indicated by red, while low expression is indicated by green. *MYCN* amplified tumors form a distinct cluster characterized by low expression levels of many miRNA loci. The 11q- tumors formed two separate clusters, which could not always be perfectly distinguished from the low stage hyperdiploid tumors. This figure and its legend was originally published by Chen and Stallings [19] in *Cancer Research* and is reproduced here with the permission of the journal.

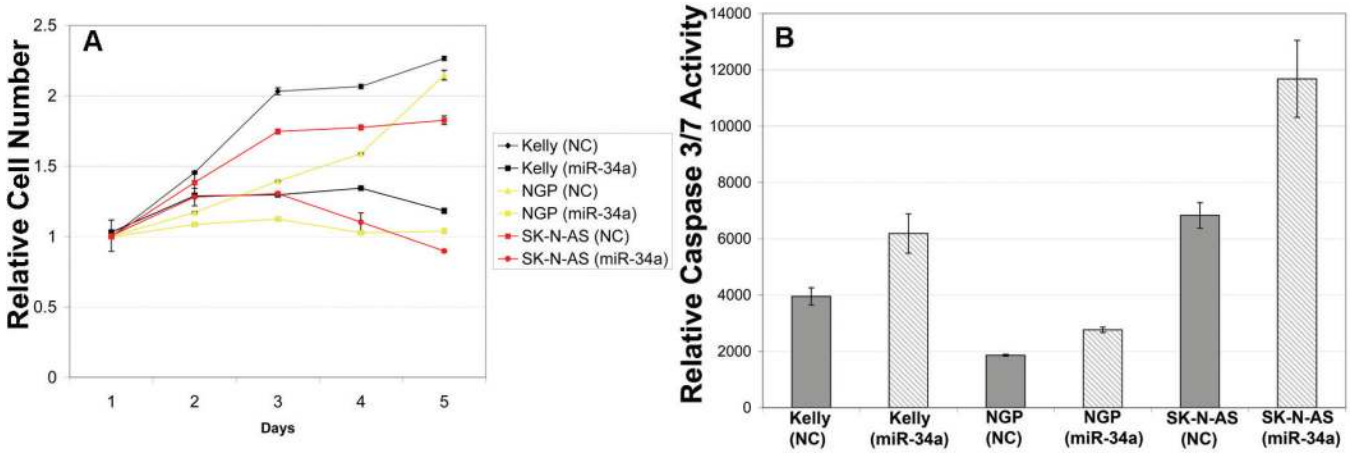

Fig. (2).

(A) MTT cell proliferation assay performed on days 1 to 5 after transfection of Kelly, NGP and SK-N-AS cells with either a pre-miR-34a molecule or a negative control oligonucleotide that does not encode for any known miRNA. Cell populations transfected with the negative control oligo had a significantly greater number of metabolically active cells than cells transfected with the pre-miR-34a. **(B)** Caspase 3/7 assay carried out on Kelly, NGP and SKN-AS cells shows that ectopic up-regulation of miR-34a (diagonal stripe) leads to an increase in caspase activity relative to a negative control oligonuclotide (gray). This figure was originally published by Welch et al [28] in *Oncogene* and is reproduced here with the permission of the journal.

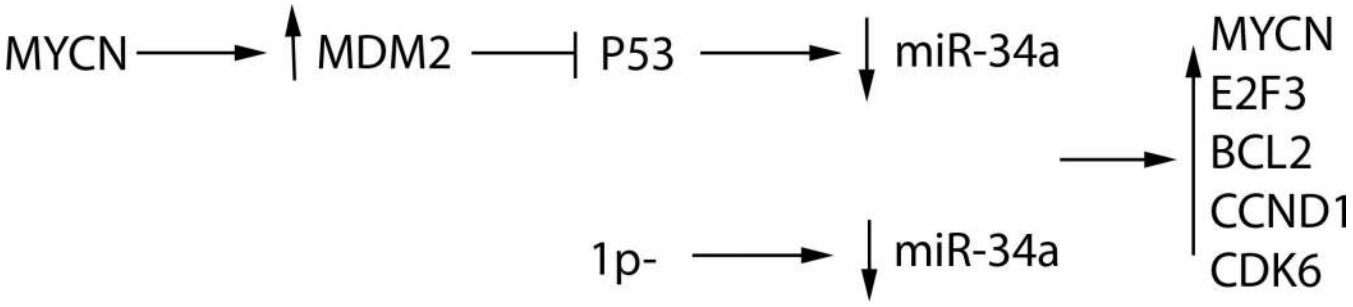

Fig. (3).
MiR34a genetic pathway. The MYCN transcription factor directly up-regulates the transcription of *MDM2*, which antagonizes the action of the p53 transcription factor. The down-regulation of P53 protein by MDM2 should indirectly cause the down-regulation of miR-34a, which requires p53 for expression. MiR-34a must be down-regulated in neuroblastoma because it directly targets *MYCN* and other proteins that either promote cell proliferation or retard apoptosis. Mir-34a is also down-regulated by chromosome 1p deletion in some tumors.