

Emerging Roles of microRNAs in Malignant Neuroblastoma

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Abstract

Discovery of microRNAs (miRNAs) as negative regulators of gene expression at post-transcriptional level has revealed a new layer of finest regulation of cell signaling mechanisms in normal development and abnormal growth. The pathogenesis in most of the tumors including malignant neuroblastoma, a childhood malignancy in most cases, is now known to be linked to aberrant expression of a wide range of miRNAs, which can be oncogenic or tumor suppressor molecules. Many miRNAs seem to be involved in avoiding differentiation and apoptotic death while driving the growth of human malignant neuroblastoma. So, modulation of expression of specific oncogenic and tumor suppressor miRNAs may provide us novel therapeutic opportunities to enhance induction of differentiation and apoptosis and also inhibition of autophagy, proliferation, multidrug resistance, migration, invasion, and metastasis in human malignant neuroblastoma.

Keywords: Apoptosis; Autophagy; Epigenetic deregulation; Malignant neuroblastoma; miRNAs

Introduction

Recent excitement in cancer research is mainly around microRNAs (miRNAs or miRs) that are small (~22 nucleotides long), highly stable, and evolutionarily conserved non-coding RNAs for finest regulation of gene expression at post-transcriptional level by moving the RNA-induced silencing complex (RISC) to the partial complementary 3' untranslated region (3' UTR) of the target messenger RNAs (mRNAs) for either their degradation or inhibition of their translation depending on degree of complementarity between the miRNAs and the 3' UTR of the target mRNAs [1]. Several kb long primary miRNA (pri-miRNA), which is derived from RNA polymerase II activity, assumes a stem-loop structure for recognition and cleavage by Drosha (a nuclear RNA III endonuclease) to generate 60-70 nucleotides long precursor miRNA (pre-miRNA) for exportation to the cytoplasm where the pre-miRNA is further processed by Dicer (another RNA III endonuclease) to the final ~22 nucleotides long miRNA duplex for attachment of its 5' end of the strand with the weakest base pairing to the RISC and movement to the 3' UTR of the target mRNA [2]. One miRNA is capable of inhibiting expression of multiple mRNAs directly or indirectly, indicating that suppression or promotion of expression and function of even a single miRNA may have profound consequences in cell signaling pathways. The main mechanism of action of a miRNA is inhibition of expression of the target gene or genes; however, in rare cases miRNAs may modulate transcription [3,4] or activate translation [5]. Because miRNAs in almost all cases kill the messenger, they are readily recognized as the negative regulators of the gene expression. It is now widely acknowledged that expression of more than 30% of human protein-coding genes is controlled by miRNAs. Emerging results suggest that miRNAs play important roles in cell cycle, proliferation, differentiation, apoptosis, and metabolism. As such, aberrant expression of miRNAs is linked to cancer development, cancer stem cells, autophagy, multidrug resistance, epithelial-mesenchymal transition (EMT), migration, invasion, and metastasis [6-8]. EMT, a process by which epithelial cells lose cell to cell contact proteins such as E-cadherin and γ -catenin to become mesenchymal cells with expression of vimentin and fibronectin, is a prerequisite for cancer metastasis [9,10]. Cells that undergo EMT are able to disconnect from the primary tumor and then migrate, leading to cancer metastasis. Depending on their targets, miRNAs can act as oncogenes and/or tumor suppressors to influence tumorigenesis. Oncogenic miRNAs kill the mRNAs of the tumor suppressor genes while tumor suppressor miRNAs kill the mRNAs of the oncogenes. Each tumor type has its

distinct miRNA profile that differs from that of other tumor types and its normal tissue counterpart. Each human malignant neuroblastoma, which is a deadly tumor mostly found in pediatric patients, also harbors highly characteristic miRNA profile [11]. Therefore, specific miRNA signatures can serve as biomarkers for diagnosis, drug response prediction, and prognosis of the malignant neuroblastoma patients [11]. Most importantly, direct or indirect manipulation of expression of specific miRNAs in human malignant neuroblastomas can provide us highly promising therapeutic opportunities to control their growth and thereby save the children.

miRNAs in Malignant Neuroblastoma

Neuroblastomas, originated from immature neuroblasts of the peripheral nervous system, are the most common extracranial solid tumors that mainly occur in infants and young children and account for about 10% of all childhood cancers and 15% of childhood death [12]. Neuroblastomas are highly noted for their high degree of heterogeneity in clinical behaviors, ranging from spontaneous regression to aggressive growth and metastasis leading to death of the patients. Deregulation of miRNAs may be an important mechanism that contributes to pathogenesis and heterogeneity of neuroblastomas [11,13]. Clinical behaviors of neuroblastomas may be considerably correlated with their specific genetic (e.g., amplifications, deletions, point mutations) abnormalities [12] and relatively rapid epigenetic (e.g., DNA methylations, histone modifications) changes [14]. Abnormally high or low levels of specific miRNAs ultimately can affect the levels of expression of target genes, causing the malignant neuroblastoma cells promote autophagy and proliferation, avoid differentiation and apoptosis, and increase angiogenesis, migration, invasion, and metastasis. Deregulation of expression of miRNAs in malignant neuroblastomas may be due to N-Myc amplification, chromosomal deletion, or abnormal epigenetic regulation [11,15]. About 25% of all

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malignant neuroblastomas are associated with N-Myc amplification and poor outcome irrespective of age [16]. Expression of N-Myc, which is a helix-loop-helix leucine zipper transcription factor, is restricted mainly to the peripheral and central nervous system [17]. N-Myc has been shown to bind to the promoter region of a wide range of miRNA genes for regulation of their expression. As many as 37 miRNAs are differentially expressed in malignant neuroblastomas with N-Myc amplification relative to tumors without N-Myc amplification [18]. Several oncogenic miRNAs (miR-17-5p, miR-92, miR-93, miR-99, miR-106a, and miR-221) are upregulated due to N-myc amplification in malignant neuroblastoma. Loss of 1p36 heterozygosity causes low expression of miR-34a, which is a potent tumor suppressor miRNA, in human malignant neuroblastoma with N-Myc amplification. Also, N-Myc is responsible for activation of methyltransferases for methylation of target genes. Promoters of miRNAs can be aberrantly modified by the deregulated epigenetic machinery for aberrant expression of miRNAs in malignant neuroblastoma. For example, a novel integrated approach recently identified 67 miRNAs under epigenomic regulation in malignant neuroblastoma [19]. Some tumor suppressor miRNAs (let-7, miR-101, and miR-202 that target N-Myc; miR-9 that targets TrkC, REST, ID2, and MMP-14; miR-34a that targets E2F3, Bcl-2, and N-Myc; miR-340 that targets SOX2; miR-184 that targets Akt2; and miR-335 that targets MAPK1, LRG1, and ROCK1) in malignant neuroblastoma are silenced by aberrant DNA methylation or histone modification [20]. Therefore, epigenetic inactivation of tumor suppressor miRNAs is currently recognized as a major hallmark of malignant neuroblastoma, without any notable opposition yet to this concept.

Inhibition of Oncogenic miRNAs in Malignant Neuroblastoma

Inhibition of expression of powerful and specific oncogenic miRNAs can be an exciting therapeutic strategy for controlling growth of malignant neuroblastoma. Because aberrant expression of oncogenic miRNAs in malignant neuroblastoma play highly crucial regulatory roles in maintaining neuroblastoma stem cells (NSCs) and tumorigenesis [20-22], it is very obvious that oncogenic miRNAs will be useful not only in diagnosis but also in designing novel therapy for controlling growth of NSCs as well as of other neuroblastoma cells in the tumors. Chemically modified antago-miRNAs or anti-miRNAs (antisense oligonucleotides as miRNA inhibitors) can be successfully used for decreasing abundance and activity of powerful oncogenic miRNAs in malignant neuroblastoma cells in culture and animal models. Growth of malignant neuroblastoma with N-Myc amplification is linked to transactivation of miR-17-5p-92 cluster that inhibits translation of p21 (a negative regulator of cell cycle by inhibiting a broad range of cyclin/Cdk complexes) and Bim (Bcl-2 interacting mediator of cell death, which is a potent pro-apoptotic BH3-only protein that binds with high affinity to all pro-survival Bcl-2 proteins) [23]. Antago-miR-17-5p treatment abolished growth of therapy-resistant human malignant neuroblastoma in vitro as well as *in vivo* due to upregulation of p21 and Bim leading to inhibition of cell cycle and induction of apoptosis, respectively [23]. Use of the enhanced green fluorescent protein reporter construct carrying the 3'UTR of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) identified RECK as the direct target of oncogenic miR-15a to induce expression of matrixmetalloproteinase-9 (MMP-9), providing the new insights into the characteristics of the miR-15a-RECK-MMP-9 axis in promoting migration and invasion of malignant neuroblastoma [24]. So, suppression of this oncogenic miR-15a upregulated expression of

RECK and significantly decreased MMP-9 and migration of malignant neuroblastoma cell lines [24].

Induction of Tumor Suppressor miRNAs in Malignant Neuroblastoma

Induction of expression of poorly present or re-expression of totally absent tumor suppressor miRNAs can be another important avenue to control the growth of malignant neuroblastoma. Enhancement of biogenesis of tumor suppressor miRNAs can be an ideal approach for development new drugs for inhibition of tumorigenesis. Tumor suppressor miRNAs may be over expressed and also re-expressed in human malignant neuroblastoma using synthetic miRNA mimics or miRNA mammalian expression vectors that carry either a pre-miRNA sequence or an artificial miRNA hairpin sequence. For example, functional analysis following transfection of miR-34a mimics showed targeted degradation of mRNAs of Bcl-2 and N-Myc, cell cycle arrest, and induction of apoptosis in human malignant neuroblastoma cell lines with 1p36 hemizygous deletion [25]. Also, functional data from miR-184 ectopic over expression using a pre-miR-184 expression vector demonstrated that miR-184 directly targeted and degraded mRNA of Akt2, a major downstream effector of the phosphatidylinositol 3-kinase (PI3K) pro-survival pathway, for significant reduction of tumor growth and increase in overall survival in an orthotopic mouse model of malignant neuroblastoma [26]. The tumor suppressor miR-128, which is upregulated during all-*trans* retinoic acid mediated differentiation of human malignant neuroblastoma SH-SY5Y cells, inhibits expression of Reelin (a glycoprotein that plays a role as a guide for migration) and DCX (doublecortin located on chromosome X, which is a microtubule-associated protein required for neuroblastic migration) to reduce neuroblastoma cell motility and invasiveness [27]. Also, ectopic overexpression of miR-128 using the DpA-miR-128 plasmid vector (encoding for miR-128) suppressed expression of Reelin and DCX and thereby reduced motility, invasiveness, and growth of neuroblastoma cells [27].

Activation of Epigenetically Silenced Tumor Suppressor miRNAs in Malignant Neuroblastoma

Activation of epigenetically inactivated tumor suppressor miRNAs may also provide therapeutic opportunities in malignant neuroblastoma. Recently, it has been shown that epigenetically silenced miRNAs highly contribute to pathogenesis in malignant neuroblastoma [19]. It is now known that N-Myc amplification in malignant neuroblastoma transcriptionally down regulate epigenetically controlled tumor suppressor miRNAs. A recent study reports the existence of LIN28B-let-7-N-Myc axis, in which LIN28B down regulates the tumor suppressor let-7 to upregulate N-Myc and drive the growth of malignant neuroblastoma [28]. Also another recent study demonstrates that N-Myc transcriptionally down regulates the epigenetically controlled miR-335 (which is a tumor suppressor miRNA), overexpression of which targets multiple genes in the TGF- β non-canonical pathway leading to inhibition of migration and invasion of malignant neuroblastoma cells [29]. Therefore, therapeutic approaches can be designed to modulate expression of the epigenetically regulated miRNA genes. Targeting epigenetic regulators can give some advantages: epigenetic modifications are easily reversible and responsive to specific chemotherapeutic agents. For example, low doses of DNA methyltransferase inhibitors such as azacitidine (AZA) and 5-aza-2'-deoxycytidine (DAC) demonstrate therapeutic effects in cancer patients [30,31]. However, high doses of the epigenetic inhibitors (AZA and DAC) have no effect on epigenome and can

cause extreme toxicities [32,33]. Low concentrations of AZA or DAC can reduce self-renewal of the tumor cells both in culture and animal models, maintaining global DNA demethylation and re-expression of important tumor suppressor genes [34]. All these studies strongly and clearly suggest the enormous potential of epigenetic therapy for re-expression of the tumor suppressor miRNA genes in human malignant neuroblastoma.

Modulation of Expression of a miRNA to Enhance Efficacy of a Therapeutic Agent in Malignant Neuroblastoma

In a combination setting, suppression of an important oncogenic miRNA or overexpression of a potential tumor suppressor miRNA can act additively or synergistically with a natural or synthetic chemotherapeutic agent for increasing its anti-tumor effects in malignant neuroblastoma. Plant-derived flavonoids, which specifically target malignant cells, have shown efficacy in inducing cysteine proteases for apoptosis in human malignant neuroblastoma cells [35]. However, combination therapy with a retinoid and a flavonoid in human malignant neuroblastoma very effectively inhibit the overall pro-survival mechanism autophagy [36] and also induce differentiation and apoptosis *in vitro* [37] and *in vivo* [38,39]. Controlling the growth of human malignant neuroblastoma with combination of retinoid and flavonoid involves alterations in expression of specific oncogenic miRNAs and tumor suppressor miRNAs [40]. It is now widely recognized that different plant-derived natural compounds such as curcumin, flavonoids, resveratrol do down regulate oncogenic miRNAs and upregulate tumor suppressor miRNAs in human malignant cells for inhibition of cell growth, induction of apoptosis, reversal of EMT, or enhancement of anti-cancer effects of conventional and natural cancer therapeutics [41,42]. Over expression of the tumor suppressor miR-7-1 potentiated the anti-tumor properties of flavonoids in human malignant neuroblastoma cell lines [42]. Also, overexpression of the tumor suppressor miR-138 increased anti-tumor effects of another flavonoid in human malignant neuroblastoma *in vitro* and *in vivo* [43]. Distinct mechanisms of drug resistance in malignant neuroblastoma may be associated with high levels of the anti-apoptotic protein Bcl-2 and also the tyrosine-related kinase B (TrkB) receptor that activates PI3k/Akt pro-survival pathway. Use of luciferase reporter assays indicated that the tumor suppressor miR-204 could directly target the 3'UTR of Bcl-2 and TrkB mRNAs and therefore transfection of malignant neuroblastoma cells with miR-204 mimics showed significant increase in sensitivity to cisplatin and etoposide for induction of apoptosis [44]. Modulation of expression of specific miRNAs have also been helpful in increasing chemosensitivity in other malignancy [45], indicating the right direction and the huge promise of targeting miRNAs as a therapeutic strategy. Therefore, investigations for modulation of expression of miRNA should be continued and accelerated to increase anti-tumor effects of natural and synthetic therapeutic agents in malignant neuroblastoma cells *in vitro* as well as *in vivo*.

Conclusion

In conclusion, recent studies on the whole RNA transcriptome have revealed complex regulatory networks of the miRNA-to-mRNA interactions whose effects now appear to be more pervasive than previously thought in many human malignancies including malignant neuroblastoma. Tactful manipulation of expression of specific miRNAs, more precisely suppression of a powerful oncogenic miRNA and overexpression of a poorly expressed tumor suppressor miRNA,

may provide great advantages to an experimental therapeutic agent for enhancement of its anti-tumor effects in malignant neuroblastoma in preclinical studies. Validation of mechanisms of efficacy and perfection of delivery methods of these miRNA based therapeutic approaches in both ecotopic and orthotopic animal models of human malignant neuroblastoma and lack of severe side effects will hopefully prompt clinical trials of miRNA based therapeutic strategies for treatment of malignant neuroblastoma in pediatric patients in the near future. Currently, a cutting-edge topic of research is miRNA in all malignant diseases. Studies on miRNAs in many other malignancies are being reported at an exponential rate every year, while only less than 150 studies so far have been reported on the emerging roles of miRNAs in malignant neuroblastoma. So, this status of the field indicates that further studies in the future will reveal the involvement of many other miRNAs in the life and death of human malignant neuroblastoma.

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