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Targeting MYCN IRES in *MYCN*-amplified neuroblastoma with *miR-375* inhibits tumor growth and sensitizes tumor cells to radiation

Hailong Zhang, Tao Liu, Sha Yi, Lubing Gu, Muxiang Zhou**

Department of Pediatrics and Aflac Cancer and Blood Disorders Center, Emory University School of Medicine, Atlanta, GA 30322, USA

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ABSTRACT

The *MYCN* oncogene is amplified in 20% of neuroblastomas, leading to its overexpression at both the mRNA and protein levels. MYCN overexpression is strongly associated with advanced disease stage, rapid tumor progression and a worse prognosis. In the present study, we identified microRNA-375 (*miR-375*) as a negative regulator of *MYCN*: enforced expression of miR-375 inhibited *MYCN*-amplified neuroblastoma *in vitro* and *in vivo*. Upon searching the website *miRbase* for possible *miR-375* binding sites within the whole *MYCN* mRNA, we found that the *MYCN* 5′-UTR had significant sequence complementarity to *miR-375*, yet no complementary sequences existed within the *MYCN* 3′-UTR and coding regions. Enforced overexpression of *miR-375* efficiently inhibited *MYCN* mRNA translation and protein synthesis, via an *IRES*-dependent mechanism. In athymic nude mouse model with human *MYCN*-amplified neuroblastoma, MYCN downregulation by *miR-375* led to inhibition of tumor cell growth and tumorigenicity. In particular, *miR-375*-regulated inhibition of *MYCN* translation was enhanced when *MYCN*-amplified neuroblastoma cells were exposed to stress stimulation, such as ionizing irradiation (IR), resulting in a remarkable increase in the neuroblastoma's sensitivity to IR-induced cell death. Our results identified a novel mechanism by which IRES-dependent translation of *MYCN* is repressed by *miR-375*, particularly during cellular stress, highlighting a potential anticancer strategy: the development of *miR-375* as a novel therapeutic agent to treat *MYCN*-amplified neuroblastoma.

1. Introduction

Neuroblastoma (NB), the most common extracranial solid tumor seen in children, is a cancer of the peripheral nervous system. NB has great variability in clinical outcome: Tumors can regress spontaneously or progress relentlessly, despite intensive treatment. Amplification of the *MYCN* gene, which occurs in about 20% of primary tumors, is an important factor predicting a poor prognosis in NB, as it correlates strongly with advanced-stage disease and treatment failure (Maris, 2010; Mathew et al., 2001). Like other members of the *Myc* family, MYCN is a transcriptional regulator that appears to play a

* *Corresponding authors*. Division of Pediatric Hematology/Oncology, Emory University School of Medicine, 1760 Haygood Drive, Atlanta, GA 30322, USA. Tel.: +1 404 727 1426; fax: +1 404 727 4455.
E-mail addresses: lbgu@emory.edu (L. Gu), mzhou@emory.edu (M. Zhou).
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critical role in controlling cell physiology, including cell proliferation and apoptosis. MYCN co-operates to transform primary cells, makes established cell lines exhibit tumorigenicity, and initiates tumorigenesis in genetically-engineered mice; thus, it demonstrates oncogenic potential (Weiss et al., 1997). In fact, MYCN protein expression increases correlate directly with both NB growth potential and the development of drug resistance (Gogolin et al., 2010; Ho et al., 2002; Hogarty, 2003; Negroni et al., 1991; Schweigerer et al., 1990).

The quantity of MYCN expressed in NB is not absolutely associated with the amplified gene copy numbers (Matthay, 2000; Tang et al., 2006); therefore, the tumor-promoting and anti-apoptotic properties of MYCN in NB may also depend on other cellular signals that regulate MYCN expression. It is known that MYCN expression is highly regulated at the translational level. Translation of mRNA can be initiated either by a cap-dependent mechanism or by internal ribosome entry, where ribosomes are directly recruited to structured regions of mRNA, known as internal ribosome entry segment (IRES), residing within the 5′-untranslated regions (5′-UTR) of mRNA. IRES elements are found mainly in mRNAs that regulate gene expression during development, differentiation, cell growth and apoptosis (Bonnal et al., 2003). In particular, IRES activity is increased under conditions where cap-dependent protein synthesis becomes greatly reduced, such as upon cellular stress and DNA damage, whereupon IRES will initiate translation of proteins that protect cells from stress (Komar and Hatzoglou, 2005). The *MYCN* 5′-UTR contains IRES, which previous studies show is highly activated in NB, even in the unstressed cells (Jopling and Willis, 2001).

The miRNAs are small, non-protein-coding RNAs that profoundly affect an array of normal biological processes and they play important roles in cancer, by regulating the expression of various oncogenes and tumor suppressors (Caldas and Brenton, 2005; Calin and Croce, 2006; Liu et al., 2010). Almost all studies describe miRNA modulation of gene expression as occurring by its binding to the 3′-UTR of target mRNA and by its promotion of mRNA degradation, inhibiting translation. For example, the *miR-34a*, *let-7* and *miR-101* were reported to be able to bind to *MYCN* 3′-UTR to inhibit MYCN mRNA translation (Buechner et al., 2011; Wei et al., 2008). Although miRNAs also appear to regulate IRES activity within the 5′-UTR (Petersen et al., 2006), to date the activity was only studied in the *HCV* virus (Diaz-Toledano et al., 2009). For the present study, we were interested in investigating whether the activity of human *MYCN* IRES is possibly regulated by existing miRNAs and, if so, in testing our new hypothesis that targeting the *MYCN* IRES with miRNA might be a useful intervention for altering the progression of MYCN-overexpressing NB.

2. Results

2.1. *Expression of* miR-375 *in NB cell lines and potential miR-375 binding locales within the MYCN mRNA*

We tested *miR-375* expression levels by qRT-PCR in 8 NB cell lines including 4 with *MYCN* gene amplification and 4 without *MYCN* amplification. The expression levels of *miR-375* were widely ranged in about 10-fold differences from line to line in the 8 lines studied. The *miR-375* expression in these cell lines seems to associate with the *MYCN* status. As seen in Figures 1A and 3 of the 4 non-*MYCN*-amplified NB lines and none of the 4 *MYCN*-amplified lines expressed high levels of *miR-375* (larger than 5 times as compared with normal cells). We employed *miRBase* (http://www.mirbase.org) to search for possible binding sites of *miR-375* within the whole *MYCN* mRNA. There were no complementary sequences of *miR-375* within the *MYCN* 3′-UTR and coding regions, but the *MYCN* 5′-UTR had significant sequence complementarity (−1 to −10, -44 to −61, and −245 to −252) to *miR-375* (see arrows, Figure 1B). The secondary structure of the *MYCN* 5′-UTR (Figure 1B) was predicted by the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and used to calculate the minimum free energy (MFE). The colors indicate the propensity of the individual nucleotides to participate in base pairs and whether or not a predicted base pair is well determined: red gives the highest probability, blue-violet the lowest probability. The 3′ end sequences of the *MYCN* 5′-UTR, which is crucial for the IRES activity, revealed a less stable secondary structure with a relatively high MFE and lower base pair probability, suggesting that the affinity of *miR-375* binding to this region is higher. We also searched for other known miRNAs that complement with the *MYCN* 5′-UTR, and found that *miR-141* also had significant sequence complementarity (−123 to −128, −248 to −254, and −288 to −294) (data not shown).

2.2. *Identification of IRES in the* MYCN 5′-*UTR*

Previous studies report that *MYCN* translation is initiated via IRES, within the *MYCN* 5′-UTR, and that *MYCN* IRES displays enhanced activity in neuroblastoma (Jopling and Willis, 2001). Because we found that the *MYCN* 5′-UTR contains sequences complementary to *miR-375* and *miR-141*, we evaluated whether *miR-375* and *miR-141* have roles in regulating the *MYCN* IRES activity. We first performed deletion mapping of the *MYCN* 5′-UTR (Figure 2A) to identify the core IRES region and to see whether that region has sequences complementary to *miR-375* and *miR-141*. We generated dicistronic plasmids containing a series of 5′ or 3′ deleted fragments (Figure 2B) of the −300 to −1 sequence of *MYCN* 5′-UTR. Each of these plasmids was transfected into an NB cell line, SK-N-SH. Upon assessment of their luciferase activities, we identified that the core IRES region contained 100 bases, located immediately upstream of the first codon (−1 to −100). As shown in Figure 2C, the construct (−100 to −1) expressed maximal FL luciferase activity, similar to that of the full-length fragment from −300 to −1, while the area −70 to −1 showed a significant reduction of FL activity. The −300 to −100 construct showed no FL activity, which further confirmed that the sequence between −100 and −1 is critical for *MYCN* IRES activity.

2.3. MiR-375 *inhibits* MYCN *IRES activity and* MYCN *translation*

We performed co-transfection and reporter assays in SK-N-SH cells, using dicistronic plasmids containing the 100-nt *MYCN* IRES plus *miR-375*, *miR-141* or control miRNA; and using either

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