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C-Myc negatively controls the tumor suppressor PTEN by upregulating miR-26a in glioblastoma multiforme cells

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ABSTRACT

The c-Myc oncogene is amplified in many tumor types. It is an important regulator of cell proliferation and has been linked to altered miRNA expression, suggesting that c-Myc-regulated miRNAs might contribute to tumor progression. Although miR-26a has been reported to be upregulated in glioblastoma multiforme (GBM), the mechanism has not been established. We have shown that ectopic expression of miR-26a influenced cell proliferation by targeting *PTEN*, a tumor suppressor gene that is inactivated in many common malignancies, including GBM. Our findings suggest that c-Myc modulates genes associated with oncogenesis in GBM through deregulation of miRNAs *via* the c-Myc–miR-26a–PTEN signaling pathway. This may be of clinical relevance.

1. Introduction

C-Myc is an oncogene that acts as a transcriptional regulator and is overexpressed in many types of human cancers [1–6]. Along with its binding partner Max, it is known to regulate transcription in many protein-coding genes [1,7,8], and activates other oncogenes and inhibits tumor suppressor genes [1]. Although studies have shown that c-Myc plays a key role in glioma proliferation by regulating genes associated with aberrant cell proliferation [9,10], the molecular mechanisms remain to be fully established.

MicroRNAs (miRNA) are small non-coding RNAs, 19–24 nucleotides in length, that target genes by binding to partially complementary sites in the 3′-untranslated region (3′-UTR) of their cognate mRNA [12,13]. Recent studies have shown that regulation of miRNAs by c-Myc confers tumor cells with proliferative advantages [14,15]. These include miR-26a which is regarded as a potential oncomir, as it targets the tumor suppressor phosphatase and tensin homolog located on chromosome 10 (PTEN) [17], which plays a critical role in tumor cell proliferation through suppression of the AKT pathway [18–20]. MiR-26a was found to be repressed by c-Myc in aggressive B-cell lymphoma (BCL) and acute myeloid leukemia (AML) [11,16]; however, its overexpression has been reported in glioblastoma multiforme (GBM) [17]. In this study, we demonstrated that miR-26a expression in GBM cells could be directly enhanced by c-Myc, which is the converse of previous reports on BCL and AML [11,16]. In addition, we provide new insights into the interaction between c-Myc and PTEN. These findings may provide a basis for novel molecular targeted therapeutic approaches in c-Myc-induced tumors, including GBM.

2. Materials and methods

2.1. Reagents

The c-Myc inhibitor, 10058-F4, was purchased from Sigma (San Francisco, SF, USA) and utilized at a concentration of 100 μM, unless differently specified.

2.2. Cell culture

Human U87 and U251 GBM cells were obtained from the ATCC (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco-BRL, Los Angeles, CA, USA) and were incubated at 37 °C in 5% CO_2.

Abbreviations: PTEN, phosphatase and tensin homolog located on chromosome 10; GBM, glioblastoma multiforme; miRNA, microRNA; 3′-UTR, 3′-untranslated region; BCL, B-cell lymphoma; AML, acute myeloid leukemia; CCK-8, Cell Counting Kit-8.

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2.3. Construction of a lentiviral vector for c-Myc overexpression

To construct the pHR-SIN-c-Myc plasmid, c-Myc cDNA was obtained, and the following oligonucleotide primers were synthesized: forward, TTTCGTACGCTGGATTACAAGGACGACGATGAC AAGGATTTTTTTCGGGTAGTGGAAA and reverse, TTTACGCGTT-TACGCACAAGAGTTCCGTA. These were cloned into the Mlu I and Bsiw I sites in the pHR-SIN plasmid.

2.4. Cell Counting Kit-8 assay

The numbers of viable cells were determined using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan). The cells were seeded in 96-well plates (4×10^3 cells/well) and incubated overnight. CCK-8 solution was added to each well (10 μl/well), and the plates were incubated for a further 2 h. The absorbance of each well was measured using a microplate reader (Synergy HT; Bio-Tek, Winooski, VT, USA) at 450 nm.

2.5. Real-time PCR

RNA was extracted from the cells using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed using TaqMan reverse transcription reagents (Invitrogen Life Technologies) and SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols. The selected genes were normalized to U6 miRNA. GAPDH mRNA was used as an internal control. All reactions were performed in triplicate.

2.6. Western blot assay

Proteins from whole cell lysates (30 μg) were separated by 10% SDS–PAGE and electroblotted to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany). The immunoblots were incubated with antibodies to c-Myc (sc-764; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PTEN (AC22; Cell Signaling Technology, Danvers, MA, USA), p-AKT (sc-7292; Santa Cruz Biotechnology) and β-actin (Abcam, Cambridge, UK).

2.7. Chromatin immunoprecipitation (ChIP) assay

DNA-protein cross-linking was carried out by incubating the cells for 10 min at 37 °C in 1% formaldehyde. After sonication, chromatin was immunoprecipitated overnight with 10 μl of anti–c-Myc antibody (sc-764). For the negative control,

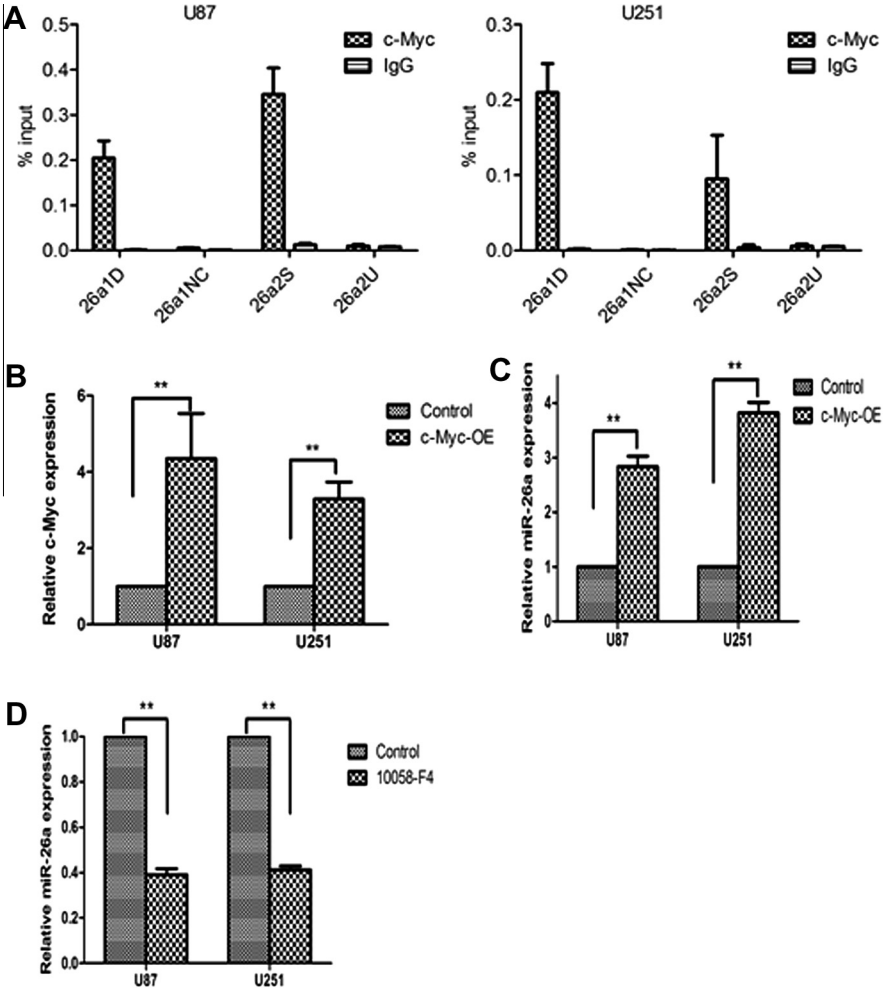

Fig. 1. C-Myc directly upregulates miR-26a expression in GBM cells. (A) The ChIP assay shows c-Myc enrichment in the pri-miR-26a1 and pri-miR-26a2 promoters in U87 and U251 cells. Furthermore, 26a1D and 26a2S represent c-Myc-binding sites with an E-box sequence; 26a1NC and 26a2U represent the negative control without the E-box sequence. IgG was used as a negative control. (B) Results from the real-time PCR assay show that stable c-Myc-overexpressing U87 and U251 cells were constructed successfully. (C and D) The effect of overexpression or inhibition of c-Myc confirms that c-Myc directly upregulates miR-26a expression in U87 and U251 cells. All experiments were performed in triplicate. $^{**}P < 0.01$.

immunoprecipitation was carried out in the absence of antibody and the amplifications were performed on the intergenic region. The relative occupancy of the immunoprecipitated factor at a locus was estimated using the comparative threshold method, using the formula: 2e(ct[input] − ct[CHIP]). Oligonucleotides for miR-26a promoter and intergenic region detection were prepared as previously described. The primer sequences used in the ChIP assay were as follows [16]:

26a1D-Chip-F: GGAGAGA CTGGGAGCGAGTGT,
26a1D-Chip-R: CAAACTCACAACCTCCCGGT;
26a2S-Chip-F: CTCCATCTGTGAGCGGCC,
26a2S-Chip-R: AAAATAGCAAAGCTCCCGACTG;
26a2U-Chip-F: CAACCTTCGAATCCCGAAAG,
26a2U-Chip-R: GAGTCCTAGGTCCGCCCAC;
26a1NC-Chip-F: AGCTACCCA GCACCAGTGTCCAA,
26a1NC-Chip-R: GGGAATTGGGGGTGGACATCACA.

Of these, 26a1D and 26a2S are located in the c-Myc-binding site E-box sequence, 26a1NC (negative control) is located in the −5 kb of pri-miR-26a1 promoter without the E-box sequence. And the 26a2U is located in the −1.2 kb of pri-miR-26a1 promoter without the E-box sequence. Both pri-miR-26a1 and pri-miR-26a2 are highly conserved in their putative promoter regions [16]. IgG was used as negative control.

2.8. Luciferase reporter assay

PCR-amplified PTEN 3′-UTR cDNA fragments containing the putative miR-26a binding site were subcloned into the pmirGLO luciferase reporter vector (Applied Biosystems). The resulting constructs, or control luciferase construct, were transfected into 293T cells. In order to determine transfection efficiency, a β-galactosidase vector was cotransfected to facilitate normalization. After 24 h of culture, the cells were exposed to either miR-26a mimic or miR-26a control oligonucleotides (100 nM) and incubated for a further 24 h. The cells were then lysed, and their luminescence was measured using a luminometer (BMG Labtech, Jena, Germany) according to the manufacturer's instructions. Experiments were repeated in triplicate.

2.9. Statistical analysis

GraphPad Prism 5.0 was used for statistical analyses. Differences between results obtained under different experimental conditions were determined by Student's *t*-test. Statistical analyses of cell proliferation were carried out by ANOVA. Experiments were repeated in triplicate. The results are given as mean and standard deviation (SD). $P < 0.05$ was considered statistically significant.

3. Results

3.1. C-Myc directly upregulated miR-26a expression in GBM cells

Sander et al. reported that c-Myc could directly regulate miR-26a in B-cell lymphoma [11]. To establish whether c-Myc played a role in the regulation of miR-26a in GBM cells, we constructed stable c-Myc-overexpressing U87 and U251 cells and performed a ChIP assay to explore the relationship between the miR-26a promoter and c-Myc. The results showed that enrichment of c-Myc was associated with the miR-26a promoter, whereas no enrichment was detected with an isotype-matched IgG antibody (Fig. 1A). This demonstrated that c-Myc can interact with the miR-26a promoter.

The enriched DNA from the immunoprecipitates was quantified by real-time PCR using primers spanning the miR-26a upstream regions at chromosome 3 and chromosome 12 (Fig. 1B). As shown in Fig. 1C, we found that c-Myc increased expression of miR-26a. To confirm these results, we used 10058-F4, a c-Myc inhibitor, to check the effect of c-Myc on the expression of miR-26a. As shown in Fig. 1D, the expression of miR-26a was reduced after treatment with 10058-F4. Taken together, these results suggested that c-Myc directly upregulated miR-26a expression in GBM cells.

3.2. MiR-26a suppressed PTEN expression by targeting the PTEN 3′-UTR

Kim et al. reported that *PTEN* was a target of miR-26a [17]. To confirm this effect in GBM cells we performed a luciferase reporter assay. A reduction in luciferase activity was observed in the U87 and U251 cells following miR-26a transfection, indicating that miR-26a mimic luciferase activity was suppressed by *PTEN* 3′-UTRs. In addition, point mutations in the putative miR-26a-binding seed region abolished the suppressive effect of *PTEN*, verifying the specificity of the target (Fig. 2A, B). These results demonstrated that *PTEN* is a specific target of miR-26a in GBM cells, in agreement with the earlier study [17].

3.3. C-Myc enhanced proliferation in GBM cells

To establish the proliferative effect of c-Myc in GBM cells, we carried out a CCK8 assay on stable c-Myc overexpressing U87 and U251 cells. As shown in Fig. 3A, the cells in the c-Myc overexpressing group had greater proliferative capacity than those in the control group, whereas the converse effect was observed following 10058-F4 treatment (Fig. 3A). These results indicated that c-Myc could enhance the proliferative ability of GBM cells, in agreement with an earlier study [10].

3.4. Evidence of a c-Myc/miR-26a/PTEN pathway in GBM cells

The c-Myc gene is frequently amplified in human GBM cells and is used as a prognostic marker in patients with GBM [21,22]. To further explore the c-Myc/PTEN relationship, we examined the

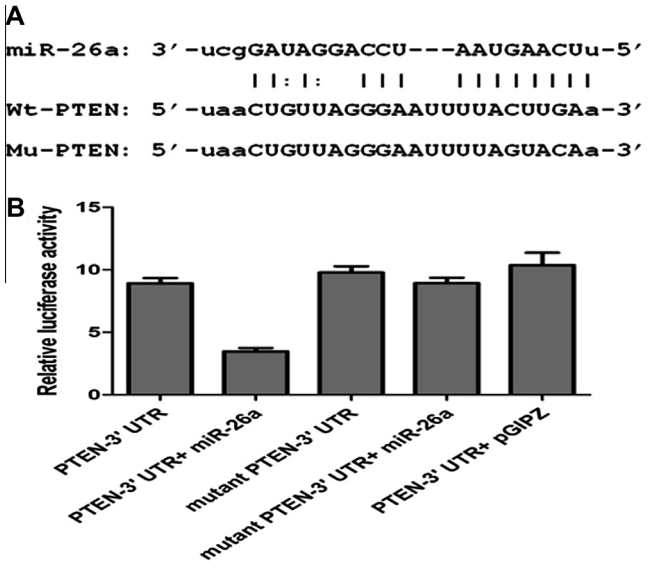

Fig. 2. MiR-26a targets the 3′-untranslated region (3′-UTR) of *PTEN*. (A) Sequence homology between miR-26a and human *PTEN* showing the miR-26a binding sites in the *PTEN* 3′-UTR. (B) The reporter assay shows the relative fold-changes in luciferase activity in U87 cells after 24 h transfection with precursor miR-26a, mutant miRNA or pGipZ scrambled control. There is a significant reduction in luciferase activity in the miR-26a–transfected cells compared to the control cells. All experiments were performed in triplicate.

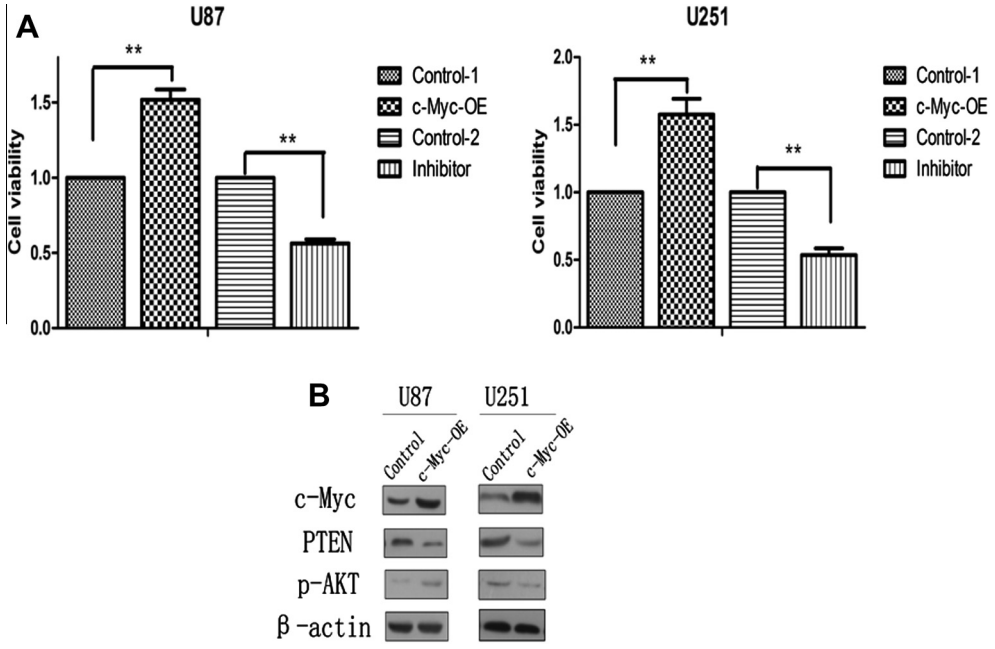

Fig. 3. C-Myc enhances the proliferative capacity of GBM cells through regulating the PTEN/AKT pathway. (A) The CCK8 assay results show that the c-Myc-overexpressing group (c-Myc-OE) has stronger proliferative capacity than the control groups and the 10058-F4 inhibitor group ($P < 0.01$). (B) Western blot assays show the effect of overexpressing or inhibiting c-Myc on PTEN and p-AKT expression in U87 and U251 cells. The results suggest that c-Myc may play a role in regulating the PTEN/AKT pathway in GBM cells. The results represent three independent experiments. $^{**}P < 0.01$.

effect of overexpressing or inhibiting c-Myc on the expression of PTEN and its downstream target p-AKT. Western blot assays showed that miR-26a expression was increased and PTEN expression was decreased in the c-Myc-overexpressing group, leading to enhancement of the downstream effector p-AKT (Figs. 1C and 3B).

4. Discussion

In this study, we have demonstrated that c-Myc can indirectly inhibit PTEN expression in GBM cells by enhancing miR-26a expression, thereby promoting proliferation. This is consistent with observations in other types of cancers [23–25].

C-Myc encodes an evolutionarily conserved basic helix-loop-helix leucine zipper transcription factor that is commonly dysregulated in cancer [26,27]. Enhanced expression of c-Myc affects key aspects of tumor biology, such as proliferation and angiogenesis, indicating that deregulation of this oncogene may be a hallmark of cancer [28]. MiRNAs are integral components of the c-Myc target gene network [26], and several studies have demonstrated that c-Myc regulation of miRNAs can lead to a significant increase in protumorigenic activity [29–32].

The aim of this study was to determine the kinetics of c-Myc-induced gliomagenesis, especially in relation to c-Myc-regulated miR-26a expression, and its impact on proliferation in GBM cell lines. By using a ChIP assay, we demonstrated that miR-26a is a c-Myc-induced miRNA in GBM cells. This is in agreement with a previous report by Kim et al. [33], who found that miR-26a was overexpressed in GBM cells, thereby acting as an oncogene. However, reports in AML and BCL have suggested that miR-26a plays a tumor suppressive role and that this action could be repressed by c-Myc [11,16]. These contradictory observations may be due to cell biology.

To further investigate the relationship between c-Myc and miR-26a, we employed miRanda software to predict miR-26a target genes for screening. *PTEN* was identified as a promising miR-26a target gene, and this was further supported by the luciferase reporter assay. PTEN is known to be a tumor suppressor and plays a critical role in tumor progression in several types of cancer through inhibition of the AKT pathway [34]. We hypothesized that c-Myc contributes to the downregulation of PTEN *via* the upregulation of miR-26a, thereby activating the AKT pathway to confer GBM cells with enhanced proliferative capacity.

By demonstrating that miR-26a expression was upregulated by c-Myc, we established a linear signaling pathway (c-Myc–miR-26a–PTEN) in which the c-Myc oncogene negatively regulated the tumor suppressor PTEN, contributing to c-Myc-induced cell proliferation. The discovery that overexpression of c-Myc could upregulate miR-26a to enhance the proliferative capability of cells suggests that inhibition of c-Myc activity could induce the converse effect, thereby decreasing proliferation in GBM cells.

In conclusion, our data indicated that miR-26a can act as an oncogene in GBM cells. This suggests that both c-Myc and miR-26a may be potential therapeutic targets for arresting proliferation in GBM.

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