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MiR-146b-5p suppresses EGFR expression and reduces in vitro migration and invasion of glioma

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Abstract

Human miR-146b-5p is located on chromosome 10q24.3. Loss of the 10q24-26 region is frequently observed in gliomas. Here, we report that miR-146b-5p suppresses expression of epidermal growth factor receptor (EGFR) in human glioblastoma cell lines. Introduction of miR-146b-5p decreases cell invasion, migration and phosphorylation of protein kinase B (AKT). MiR-146b-5p suppresses translation of EGFR, and binds to the EGFR 3'-UTR. Furthermore, analysis of U87-MG laser-capture micro-dissected cells in tumor-bearing mice indicated that expression of miR-146b-5p was inversely correlated with distance from the tumor core. These findings suggest that reconstitution of miR-146b-5p may be useful for treatment of this invasive tumor.

INTRODUCTION

MicroRNAs (miRNAs) are single-stranded 17–27 nucleotide RNA molecules that regulate gene expression by post-transcriptional silencing of target mRNAs [16]. Each miRNA can target a number of genes, and may function as oncogenes and/or tumor suppressors [2]. A growing body of evidence indicates aberrant gene expression is the main mechanism of miRNA dysfunction in cancer, with abnormal expression levels of miRNA in tumor samples compared to normal tissue [8]. Elevated EGFR expression and invasiveness are hallmarks of glioma and increase with malignancy grade [12]. Deletions on chromosome 10 are the most frequent chromosomal alteration observed in GBMs, with approximately 60 to 95% of cases exhibiting loss of heterozygosity (LOH) [10]. As human miR-146b-5p (hsa-mir-146-5p) is located within 10q24-26 (104186259-104186331+), a region of chromosomal material most frequently lost in GBM [11], we sought to test whether expression of miR-146b-5p reduces EGFR and/or invasion of two human glioma cell lines determined to have chromosome 10 LOH [5, 18], and within primary human glioblastoma cells. Based on in silico analysis (miRNAMAP [4]), the 3'-UTR of EGFR is a predicted target of miR-146b-5p. Therefore, we tested whether miR-146b-5p affected EGFR expression. Here, we report the miRNA miR-146b-5p expression is lower in U87-MG, U251 and HF66 glioma cells as compared to normal human astrocyte brain control. Transfection of these tumor cell lines with a miR-146b-5p mimic reduces expression of EGFR, as well as phosphorylation of AKT. In vitro, miR-146b-5p significantly decreased glioma invasiveness and migration. Furthermore, RT-PCR analysis of hsa-miR-146b-5p in U87-MG cells isolated by laser capture micro-

dissection (LCMD) in tumor-bearing nude mice indicated that expression of miR-146b-5p was inversely correlated with glioma invasiveness in brain.

MATERIALS AND METHODS

Cells and miRNA transfection

U87-MG and U251 cells were obtained from the American Type Culture Collection (Manassas, VA). HF66 cells were established by the Neurosurgical Department of Henry Ford Hospital [7]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human cerebral cortical astrocytes were obtained from SciencCell Research Laboratories (Carlsbad, CA). Transfection of the miRIDIAN hsa-miR-146b-5p miRNA mimic, inactive (scrambled) control cel-mir-67 (Thermo Scientific Dharmacon, IL), or pMIR-Report vectors was performed using Lipofectamine 2000 transfection reagent (Invitrogen, CA) with 300 nmol of miRNA or 1 µg/ml DNA plasmid, respectively. Transfection efficiency was verified with a siGLO DY-547 siRNA control (Dharmacon).

Western blot and Real-time RT-PCR

Western blot was performed to detect EGFR, p-EGFR (Santa Cruz, CA), AKT and p-AKT (Cell Signaling, MA) and β-actin (Santa Cruz, CA). Protein expression was measured 48 hours after transfection. Protein concentration was quantified using a BCA protein assay kit (Pierce, IL). For quantitative analysis, densitometric measurements of three blots per group were averaged. Densitometry was performed with the MCID image analysis program (Cambridge, UK). The image density (1/intensity) of each band was normalized to beta-actin, and experimental group band density was divided by the band density of control. RT-PCR for miRNAs was performed using a miR-146b-5p TaqMan MicroRNA Assay. Mirnu43 was used as a house-keeping control. Each sample was tested in triplicate and relative gene expression was determined.

Invasion and migration assays

Invasion was determined using 24-well BD Matrigel invasion chambers (8.0 µm pore size; BD Biosciences, Cowley, UK) in accordance with the manufacturer's instructions, as previously described [17]. Cells were seeded in the upper well of the invasion chamber in DMEM without serum. The lower chamber well contained DMEM supplemented with 10% FBS to stimulate cell invasion. Cells were stained with CellTracker Green (Molecular Probes, OR). Three fields of cells adhering to the lower membrane surface were counted in each well at 10× magnification (n=3 per group). Migration was measured using a scratch-wound healing assay. 48 hours post-transfection, cells were plated in 24-well plates at confluence and incubated overnight (n=4 per group). Using a 1 mL pipette tip, a scratch was drawn along the cell monolayer. Cell migration was determined in each well by the ratio of the scratch wound area devoid of cells at 24 hours to the initial scratch wound area ($\text{area}_{t=24h}/\text{area}_{t=0h}$). Cell migration experiments were conducted in complete culture medium, DMEM supplemented with 10% FBS.

Laser-capture microdissection of U87-MG glioma model in nude mouse

Athymic nude mice were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg), fixed in a stereotaxic device, and 5×10^5 U87-MG cells were injected using a 10 µL Hamilton syringe (5 µL volume). The craniectomy was covered with a film of polyvinyl chloride glued to the surrounding intact bone. Animals (n=4) were sacrificed 4 weeks post-implantation. 8 µm frozen coronal sections were used for laser-capture experiments. Using LCMD (DM6000M, Leica, Germany), we excised U87-MG xenograph cells from three distinct

regions in nude mouse brain: 1) from the center of the bulk tumor (>100 μ m from the tumor/brain border), 2) from the tumor periphery (bulk tumor mass within 100 μ m of normal brain tissue and 3) from invasive tumor (tumor cells or islets completely surrounded by brain parenchyma). Following LCMD, total RNA was isolated from tumor tissue, and using specific primers, miR-146b-5p and control mir-rnu43 miRNA was reverse-transcribed and amplified.

Cell viability and growth assays

For MTT, 2000 cells were added to each well of a 96-well plate (n=3 wells/group per time point). At 24, 48 and 72 hours, 5mg/ml MTT in PBS was added at 0.5mg/ml and cells were incubated for 4 hours. Blue formazan was dissolved in 10% SDS at 37°C overnight. Absorbance was read at 540nm. For growth assay, 10×10^3 cells were plated in each well of a 96-well plate containing culture medium. Every 24 hours, total cells in the wells were quantified using a hemacytometer. Cell counts of three wells per time point per group were averaged.

MiRNA luciferase assay

Position 1064–1085 of the EGFR 3' UTR is a predicted interaction position of miR-146b-5p. To determine if miR-146b-5p binds to the EGFR 3' UTR and inhibits translation, we cloned an 80 bp sequence containing the predicted target site or a scrambled sequence downstream of the pMIR luciferase reporter to generate pMIR-EGFR and pMIR-mut-EGFR vectors, respectively. U87-MG cells were co-transfected with these luciferase vectors and either hsa-miR-146b-5p miRNA mimic or control cel-mir-67 mimic. pMIR-Report luciferase vector alone was employed to determine transfection efficiency.

Statistical analysis

In all in vitro studies, three unique experiments were performed employing cells from different transfection experiments. Student's t-test was used for comparing means between two groups. In all experiments, a p-value of 0.05 or less was considered statistically significant.

RESULTS

MiR-146b-5p is located at 10q24.3, within a region exhibiting loss of heterozygosity in a majority of GBMs [13]. Thus, we compared the expression of miR-146b-5p in the U87-MG and U251 glioma cell lines and HF66 primary human glioblastoma cells compared to normal primary human astrocytes. Real-time PCR indicated miR-146b-5p levels in all glioma cells were significantly lower than in normal astrocyte control (Fig 1A).

MiR-146b-5p is predicted to bind to the 3' UTR of EGFR mRNA. To determine whether miR-146b-5p directly binds to EGFR mRNA, we employed a luciferase reporter expression vector system. U87-MG cells were transfected with a pMIR-Report luciferase reporter expression vector containing a cloned sequence containing the predicted miR-146b-5p binding site with in the EGFR 3' UTR (pos: 1064–1085), a sequence with mutations in the site upstream of the luciferase, or empty vector control. Cells were transfected with miR-146b-5p. 24 hours post-transfection, luciferase activity was significantly decreased in U87-MG cells transfected with both miR-146b-5p and the 1064–1085 EGFR mRNA sequence compared to mutant or pMir-Report controls (Fig. 1B). These data indicate EGFR mRNA is a functional downstream target for miR-146b-5p.

It was recently reported miR-146b-5p could inhibit expression of EGFR in breast cancer cells [6]. To test whether miR-146b-5p could alter the expression of EGFR protein in

glioma, we performed Western blot. Employing a 'scrambled' cel-mir-67 or miR-146b-5p mimic, we found EGFR protein expression was significantly reduced by miR-146b-5p compared to miRNA controls in all glioma cells (Fig. 1C). EGFR activates the phosphoinositide 3-kinase (Pi3K)/AKT pathway [3]. Here, we found phosphorylation of AKT was significantly decreased by miR-146b-5p whereas total AKT protein was unchanged. These results suggest that miR-146b-5p regulation of EGFR protein expression is due to inhibition of translation via direct binding to EGFR mRNA.

MiR-146b-5p decreases invasion of breast cancer cells in vitro and reduces lung metastasis in vivo [6]. EGFR activity promotes glioma invasiveness, and can contribute to hypoxic-induced glioma invasion [15, 17]. To determine if miR-146b-5p had a similar effect upon glioma, we performed a Matrigel invasion chamber assay. MiR-146b-5p or scrambled-miRNA transfected U87, U251, and HF66 cells that invaded through the extra-cellular protein matrix after 24 hours were imaged and counted (Fig 2A). In all groups, miR-146b-5p reduced the number of cells that invaded compared to controls (Fig. 2B). Likewise, employing a scratch wound healing assay, we tested the effect of MiR-146b-5p upon U87-MG, U251 and HF66 glioma cell migration (Fig 3A, B and C, respectively). Here, we found miR-146b-5p reduced migration of all glioma cells compared to scrambled-miRNA transfected controls (Fig. 3D). Additionally, we employed LCMD to test human miR-146b-5p expression levels in glioma cells of U87-MG xenographs in nude mouse 4 weeks post-implantation. Measuring miR-146b-5p in three distinct regions, we found miR-146b-5p expression in transplanted U87-MG was highest in the tumor core (>100µm within the bulk tumor), and lowest in invasive U87-MG cells or islets distinct from the bulk tumor mass, that were surrounded by brain parenchyma (Fig. 4A, B). Taken together, these data suggest miR-146b-5p antagonizes pathways involved in cell invasion and motility in vitro, and its expression may affect glioma invasiveness in vivo.

Expression of miR-146b-5p in U251 and U87-MG reduced expression of EGFR. EGFR activity has been reported to enhance GBM proliferation [14]. Therefore we tested whether miR-146b-5p altered the growth or viability of these cells. Employing a MTT assay, we tested U87-MG and U251 cell viability at 48, 72 and 96 hours post-transfection. Metabolism of MTT indicated a trend of decreased cell viability in U87-MG (Fig. 4C) and U251 (Fig. 4D) compared to non-transfected or scrambled miRNA-transfected controls; however, the difference in means was not considered significant based upon our selected 95% confidence interval. A similar result was observed in a growth assay, where total cell counts at 24, 48, 72, 96 and 120 hours post-transfection indicated a slight reduction in U87-MG (Fig. 4E) or U251 (Fig. 4F) cell numbers. The detected difference in means between miR-146b-5p-treated cells and control groups was considered significant after 96 hours of culture. These data suggest miR-146b-5p has a modest anti-proliferative effect upon U87-MG and U251 in vitro, but this effect was not detectable until several days in culture.

DISCUSSION

Here we demonstrate the U87MG and U251 human glioma cell lines and HF66 primary glioblastoma cells express less miR-146b-5p compared to normal human brain astrocytes. We did not determine if the miR-146b-5p genomic sequence was specifically underrepresented in these cells due to chromosomal aberrations. However, U251 and U87-MG exhibit significant deletions on chromosome 10 [5, 18], and miR-146b-5p in these cell lines was significantly less compared to normal astrocytes. Thus, LOH may be a factor in the lower miR-146b-5p expression seen in U251 and U87-MG cells. Reconstitution of miR-146b-5p activity resulted in decreased migration and invasion in vitro, and slightly reduced cell growth in culture. Using LCMD, we found that expression of miR-146b-5p negatively correlated with invasiveness of U87-MG cells in nude mouse brain. Here we did

not test if miR-146-5p could alter invasion in vivo; however our LCMD findings suggest the effect of miR-146b-5p upon glioma invasion in vitro might be relevant in vivo. In addition, we found miR-146b-5p antagonizes expression of EGFR. Our findings regarding EGFR and invasion are in accord with a recent report that miR-146b-5p decreased EGFR expression and invasion in the breast cancer cell line MDA-MB-231 [1, 6]. EGFR has been established as a potent agonist of glioma invasion [15]. In fact, it has been reported that EGFR amplification status is heterogeneous within a glioma population, and that EGFR expression is positively correlated with invasiveness [9]. EGFR activates the PI3K/AKT pathway [3], and we found transfection with miR-146b-5p significantly reduced levels of phosphorylated AKT.

Each miRNA targets a number of genes [2]. Depending upon the pathways targeted, miRNAs can function as tumor suppressors or oncogenes, and have the potential to be either depending upon the physiological context [16]. However, due to the numerous potential targets of miR-146b-5p, its inhibitory effect upon glioma invasiveness is likely an emergent phenotype resulting from alteration of possibly both pro-invasive and anti-invasive factors. Nevertheless, expression of miR-146b-5p reduced lung metastasis of MDA-MB-231 cells, suggests its anti-invasive effect is maintained in vivo [6]. Furthermore, our finding that miR-146b-5p is reduced in invasive U87-MG cells compared to those within the bulk tumor in a nude mouse model of brain tumor, implies miR-146b-5p expression may have a similar effect upon tumor invasion in glioma.

GBMs are characteristically invasive tumors. Aberrant expression of EGFR occurs in a majority of gliomas, and EGFR activity positively correlates with malignancy [14, 15]. MiR-146b-5p resulted in decreased expression of EGFR. Therefore, we suggest this miRNA may represent a novel target for therapeutic intervention in EGFR-dependent gliomas. Furthermore, as a majority of gliomas exhibit loss of heterozygosity of chromosome 10 [11, 13], the miRNA miR-146b-5p is likely under-expressed in a majority of these tumors, particularly in those of a high-malignancy grade where 10q24-26 loss is common.

Acknowledgments

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Fig. 1.

(A) miR-146b-5p expression is higher in normal human cortical astrocytes compared to U87-MG or U251 glioma cell lines, or HF66 primary human glioma cells (** $p < 0.01$ vs. human astrocytes). (B) Predicted binding site of human miR-146b-5p and the 3' UTR regions of human EGFR; MiR-146b-5p targets the predicted binding site within the EGFR mRNA 3' UTR, decreasing luciferase in U87-MG cells transfected with a luciferase reporter vector containing the predicted binding site. pMir-Report: vector control; mut-EGFR: vector containing mutated binding site; EGFR: vector containing predicted binding site. (** $p > 0.01$ compared to U87-MG transfected with vector containing a mutated EGFR site and miR-146b-5p). (C) Western blot indicates transfection with miR-146b-5p mimic decreases EGFR, and phosphorylated AKT protein in U87-MG, U251 and HF66 cells. Scrambled (cel-mir-67) had no significant effect compared to non-transfected controls. Mean band densities vs. scrambled miRNA control were determined by three experiments. (# $p < 0.05$, ## $p < 0.01$ vs. scrambled miRNA control).

Fig. 2. MiR-146b-5p reduces glioma cell invasion. (A) Representative fields ($\times 10$ magnification) showing invasive cells after 24 hour culture in Matrigel invasion chambers. Panels show U87-MG, U251 or HF66 cell invasion after transfection with miR-146b-5p or scrambled miRNA control. (B) Quantitative analysis of cell invasion experiments demonstrates that miR-146b-5p decreases U87-MG, U251 and HF66 glioma cell invasion compared to scrambled miRNA control (* $p < 0.05$, ** $p < 0.01$, vs. scrambled miRNA control).

Fig. 3. MiR-146b-5p reduces glioma cell migration. (A, B) Representative fields ($\times 4$ magnification) shows a scratch wound in U87-MG or U251 cell monolayer before and after 24 hours of culture. (C) Quantitative analysis of cell migration into the scratch area demonstrates miR-146b-5p decreases U87-MG, U251 and HF66 glioma cell migration compared to non-transfected or scrambled miRNA controls (* $p < 0.05$, ** $p < 0.01$ v.s scrambled miRNA control).

Fig. 4. (A) MiR-146b-5p expression in U87-MG xenografts in nude mouse is lower in invasive cells compared to primary tumor mass. Laser capture of tumor tissue demonstrating different regions of capture: 1) tumor core, 2) tumor boundary, 3) invasive tumor. (Subsequently stained with H&E for visualization.) (B) Real-time RT-PCR analysis of miR-146b-5p expression in U87-MG cells excised by LCMD from 4 tumor-bearing mice ($p^{**} < 0.01$ vs. tumor core). (C, D) MTT cell viability assay indicated miR-146b-5p did not significantly decrease cell viability in U87-MG (C) or U251 (D) cells 48, 72, or 96 hours post-transfection. (E, F) Cell growth assay revealed miR-146b-5p transfection resulted in reduced U87-MG (E) and U251 (F) cell numbers after 96 hours in culture (* $p < 0.05$ vs. non-transfected control, # $p < 0.05$ v.s scrambled miRNA control).