

MicroRNA-21 Is an Antiapoptotic Factor in Human Glioblastoma Cells

Jennifer A. Chan,^{1,3} Anna M. Krichevsky,² and Kenneth S. Kosik⁴

Departments of ¹Pathology and ²Neurology, Brigham and Women's Hospital and ³Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts; and ⁴Neuroscience Research Institute, University of California Santa Barbara, Santa Barbara, California

Abstract

MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate protein expression by targeting the mRNA of protein-coding genes for either cleavage or repression of translation. The roles of miRNAs in lineage determination and proliferation as well as the location of several miRNA genes at sites of translocation breakpoints or deletions has led to the speculation that miRNAs could be important factors in the development or maintenance of the neoplastic state. Here we show that the highly malignant human brain tumor, glioblastoma, strongly overexpresses a specific miRNA, miR-21. Our studies show markedly elevated miR-21 levels in human glioblastoma tumor tissues, early-passage glioblastoma cultures, and in six established glioblastoma cell lines (A172, U87, U373, LN229, LN428, and LN308) compared with nonneoplastic fetal and adult brain tissues and compared with cultured nonneoplastic glial cells. Knockdown of miR-21 in cultured glioblastoma cells triggers activation of caspases and leads to increased apoptotic cell death. Our data suggest that aberrantly expressed miR-21 may contribute to the malignant phenotype by blocking expression of critical apoptosis-related genes. (Cancer Res 2005; 65(14): 6029-33)

Introduction

The discovery of microRNAs (miRNAs) has broadened our understanding of the mechanisms that regulate gene expression with the addition of an entirely novel level of regulatory control. These small noncoding transcripts of 18 to 25 nucleotides modulate protein expression by binding to complementary or partially complementary target mRNAs and thereby targeting the mRNA for degradation or translational inhibition (1). Although knowledge of the specific mRNA targets for the ~250 to 300 known mammalian miRNAs is limited, one emerging function of this extensive regulatory network is its control over processes that underlie cell proliferation and differentiation in diverse organisms during normal development (2). In *Caenorhabditis elegans*, miRNAs regulate temporal transitions between developmental stages (3, 4). In *Drosophila*, the miRNA *bantam* both prevents apoptosis and stimulates cell proliferation by suppressing the proapoptotic gene *hid* (5). In mammals, functions for specific miRNAs have been described in the processes of hematopoietic (6) and adipocyte

differentiation (7) and insulin secretion (8). miRNAs are precisely regulated and characteristic patterns of miRNA expression appear during brain development and neuronal differentiation *in vitro* (9–11). A potential role for miRNAs in malignancy has been suggested by the location of the genes for several miRNAs at sites of translocation breakpoints or deletions linked to human leukemias (12). Indeed, altered miRNA expression has now been reported in leukemia, lung cancer, and colon cancer (13–15). The functional significance of these changes, however, has yet to be addressed. Here we examined the expression of miR-21 in glioblastoma multiforme. We show that high expression of miR-21 is a common feature of glioblastoma multiforme and that miR-21 can function as an antiapoptotic factor in cultured glioblastoma multiforme cells.

Materials and Methods

Human tissue samples. Fresh frozen human nonneoplastic brain tissue and human tumor samples were obtained from the Department of Pathology at Brigham and Women's Hospital. All human materials were used in accordance with the policies of institutional review board at Brigham and Women's Hospital.

Cell lines and culturing conditions. Early-passage (passage 3) cultures from four independent human high-grade gliomas were a generous gift from Dr. David Louis (Massachusetts General Hospital). From each of the four high-grade gliomas, three cultures were established to give a total of 12 early-passage cultures (passage 3) used in our studies. Human glioblastoma cell lines A172, U87, LN229, U373, LN428, and LN308 were kindly donated by Drs. Azad Bonni and Rosalind Segal (Harvard University). All glioblastoma cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Undifferentiated P19 mouse embryonal carcinoma cells were cultured in α MEM supplemented with 10% FBS and were routinely passaged at 2- to 3-day intervals. Primary rat cortical astrocyte cultures were prepared from the cerebral cortices of newborn pups according to established protocols (16).

MicroRNA array and Northern blot analysis. Total RNA from human tissue samples and cell lines was isolated with Trizol reagent (Invitrogen, Carlsbad, CA). Oligonucleotide arrays were printed with tri-mer oligonucleotide probes (antisense to miRNAs) specific for 180 human and mouse miRNAs on GeneScreen Plus (NEN) membranes, and miRNA expression profiling was done and analyzed as described previously (9). To ensure accuracy of the hybridizations, each labeled RNA sample was hybridized with three separate membranes. Northern blots were done with 15 μ g of total RNA as previously described (9).

Oligonucleotide transfection. 2'-O-methyl (2'-OMe-) oligonucleotides and locked nucleic acid (LNA/DNA) oligonucleotides were chemically synthesized by Integrated DNA Technologies (Coralville, IA). 2'-O-Methyl oligos were composed entirely of 2'-O-methyl bases and had the following sequences: 2'OMe-EGFP antisense 5'-AAGGCAAGCUGACCCUGAAGU-3' and 2'OMe-miR-21 5'-GUCAACAUCAGUCUGUAAGCUA-3'. LNA/DNA oligos contained locked nucleic acids at eight consecutive centrally located bases (indicated by underline) and had the following sequences: LNA-miR-21 5'-TCAACATCAGTCTGATAAGCTA-3', LNA-scrambled 5'-CATTAATGTCG-GACAACCTCAAT-3', LNA-miR124a 5'-GGCATTCCACCGCTGCCTTA-3', and LNA-miR125b 5'-TCACAAGTTAGGTCTCAGGGA-3'.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

J.A. Chan and A.M. Krichevsky contributed equally to this work.

The authors have no conflicting interests to report.

Requests for reprints: Anna M. Krichevsky, Neurology, Brigham and Women's Hospital, Harvard Medical School, 4 Blackfan Circle HIM 760, Boston, MA 02115. Phone: 617-525-5195; Fax: 617-525-5305; E-mail: krichevsky@end.bwh.harvard.edu.

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Cells were transfected using LipofectAMINE 2000 reagent (Invitrogen) 24 hours after plating. Transfection complexes were prepared according to the manufacturer's instructions and added directly to the cells to a final oligonucleotide concentration of 10 nmol/L. Transfection medium was

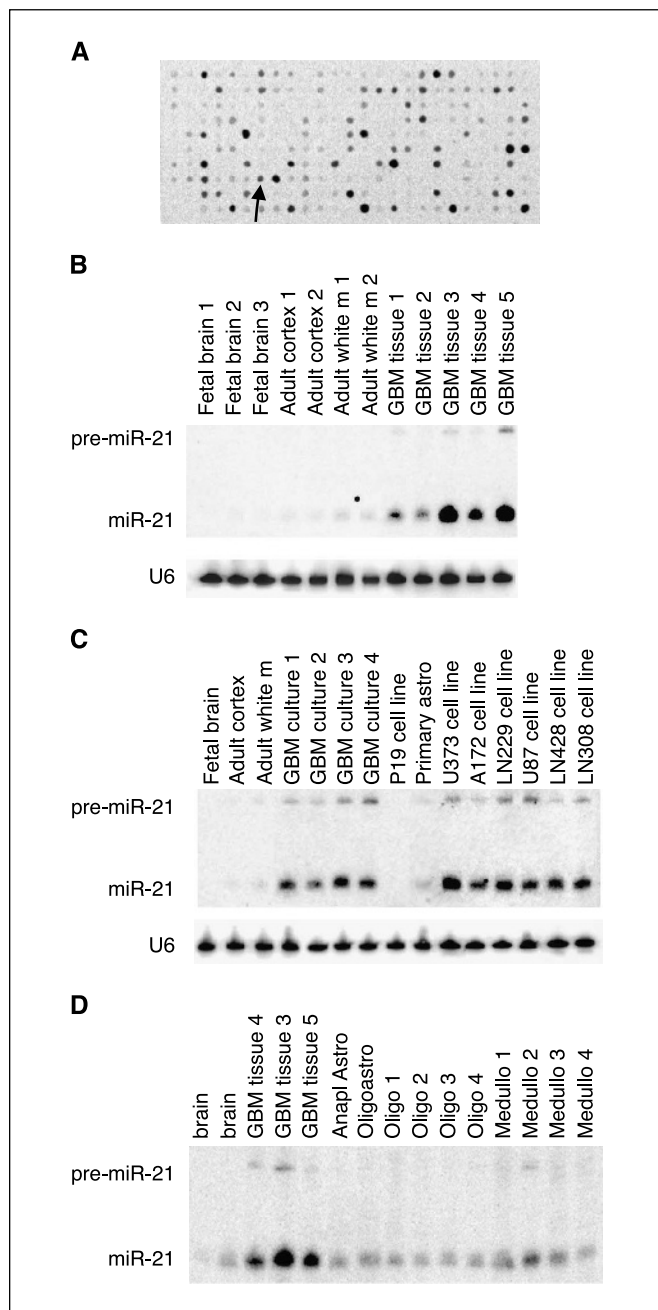


Figure 1. miR-21 is overexpressed in glioblastoma tissues and cells. **A**, representative view of glioblastoma multiforme (GBM) RNA hybridized to an oligonucleotide array containing multiple control probes and probes specific for 180 miRNAs. Position of the miR-21 probe (arrow). **B**, Northern blot showing expression of miR-21 in nonneoplastic human control tissues (fetal brain, adult cortex, and adult white matter) and tissue from five human glioblastoma samples (GBM tissues 1-5). **C**, Northern blot showing expression of miR-21 in control tissues (fetal brain, adult cortex, adult white matter), control cell cultures (P19 cell line and primary astrocytes), early-passage cultures from four high-grade gliomas (GBM cultures 1-4), and six established glioblastoma model cell lines (U373, A172, LN229, U87, LN428, LN308). Blots were reprobed for U6 snRNA as a loading control. **D**, Northern blot showing miR-21 expression in control tissues, glioblastoma multiformes, one anaplastic astrocytoma, one oligoastrocytoma, four oligodendrogliomas, and four medulloblastomas.

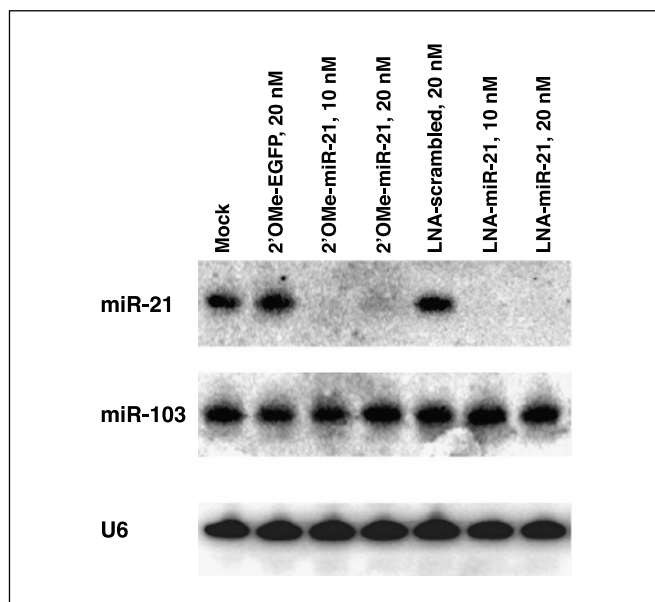


Figure 2. Sequence-specific knockdown of miR-21 by 2'-O-methyl- and DNA/LNA-oligonucleotides. Northern blot showing miR-21 expression after transfection with vehicle control (Mock), an irrelevant 2'-O-methyl-oligonucleotide (2'OMe-EGFP), a 2'-O-methyl-oligonucleotide complementary to miR-21 (2'OMe-miR21), an irrelevant DNA/LNA-oligonucleotide (LNA-scrambled), and a DNA/LNA-oligonucleotide complementary to miR-21 (LNA-miR-21). The same blot was reprobed for miR-103 and U6 snRNA as specificity and loading controls.

replaced 8 hours post-transfection. For studies of repeated transfection, surviving cells at 72 hours post-transfection were collected, replated, and transfected again 24 hours after replating (96 hours after initial transfection).

Cell number assay. A172, U87, LN229, and LN308 cells were plated at 3×10^3 cells per well in 96-well plates with five replicate wells for each condition, transfected, and assayed 48 hours post-transfection. Metabolic activity of the cells was determined using a luminescent ATP-based assay (CellTiter GLO, Promega, Madison, WI) according to the manufacturer's instructions. Results were read with a fluorescent plate reader with a read time of 1 second per well.

Apoptosis assays. For detection of caspase-3 and caspase-7 activation, A172, U87, LN229, and LN308 cells were plated in replicates of five in 96-well plates, transfected as described above and analyzed using ApoONE Homogeneous Caspase 3/7 Assay (Promega) according to the manufacturer's instructions. Samples were read after 1 hour of incubation with the caspase substrate on a fluorescent plate reader using wavelengths of 480 and 535 for excitation and emission, respectively.

For labeling nuclei of apoptotic cells, A172 cells were plated on glass coverslips in 24-well plates, transfected, fixed in 4% paraformaldehyde 48 hours post-transfection, and terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining was done using the DeadEnd fluorometric TUNEL system (Promega) according to the manufacturer's protocol. The number of TUNEL-positive cells was divided by the number of 4',6-diamidino-2-phenylindole-stained cells to yield the percent apoptotic nuclei. Two $40\times$ objective fields containing ~ 300 cells each were counted per coverslip, with three coverslips analyzed per condition.

Results and Discussion

We first identified miR-21 as a candidate miRNA dysregulated in gliomas during a screen for miRNA expression using miRNA arrays. We used an oligonucleotide array prepared in our laboratory (12) capable of determining expression patterns of 180 mammalian

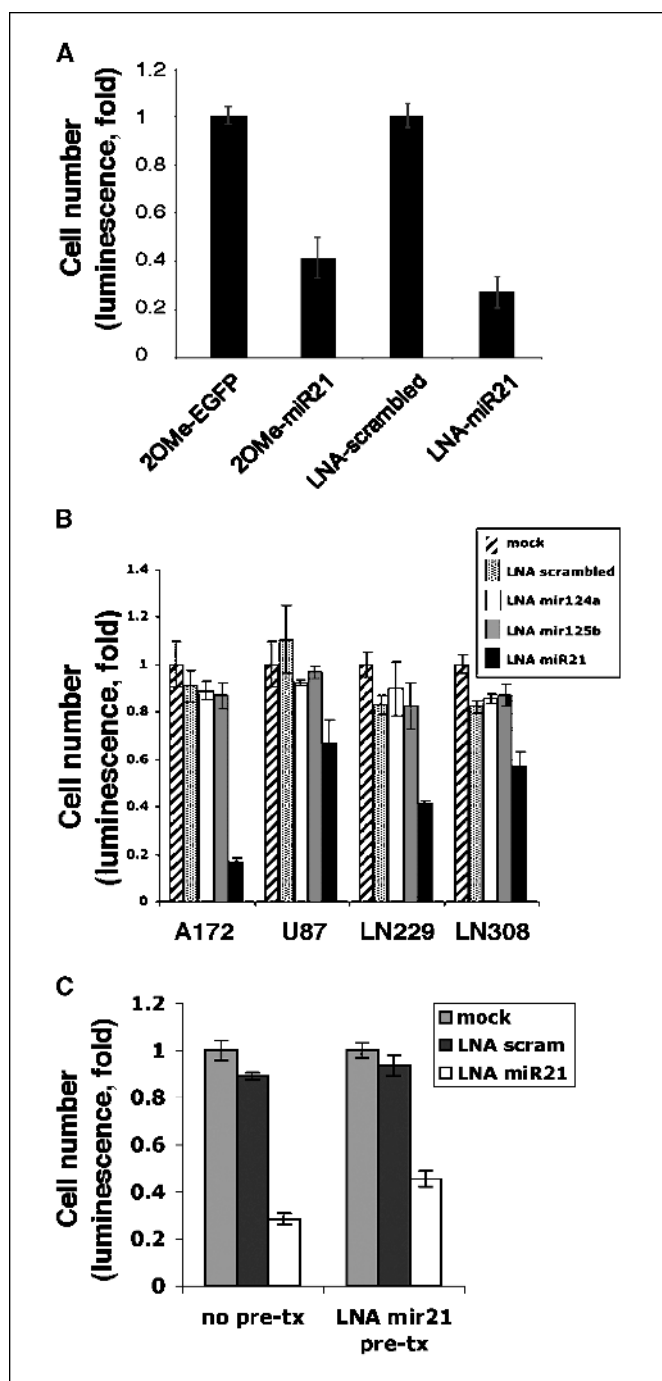


Figure 3. Inhibition of miR-21 function leads to decreased cell number. *A*, cell number assay done on A172 cells after transfection with the indicated oligonucleotides. *B*, effect of miR-21 knockdown on cell number in several glioblastoma cell lines. *C*, effect of miR-21 knockdown by LNA-miR21 on naive A172 cells versus A172 cells that survived previous treatment with LNA-miR21. *A*, columns, mean (calculated from three separate experiments of five replicates each); bars, SE. *B* and *C*, columns, mean of four replicates in one experiment; bars, SD. $P < 0.01$ (z test) for all comparisons of LNA-miR-21 and 2'OMe-miR-21 with their respective controls in (*A*) and (*B*).

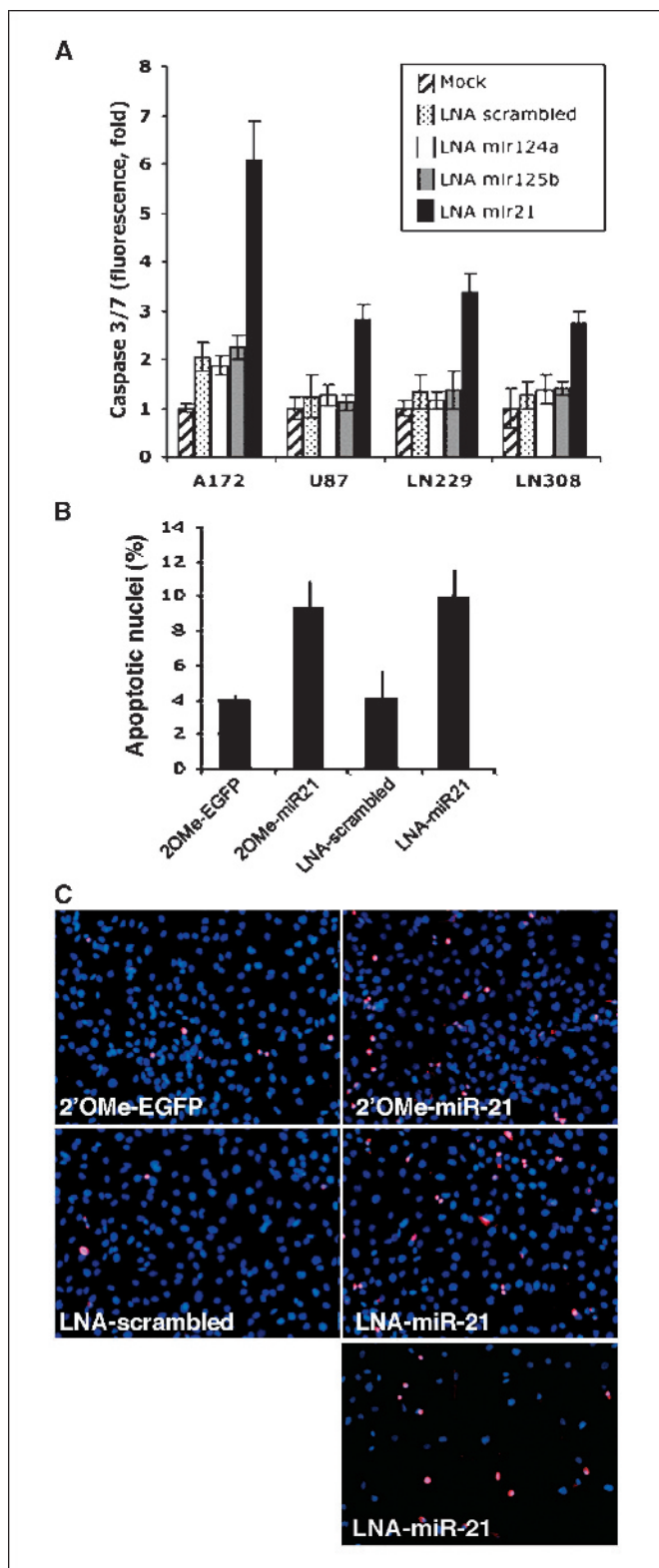
miRNAs (Fig. 1A; Supplementary Data 1). In our analyses of RNA extracted from three primary high-grade gliomas and eight nonneoplastic fetal and adult brain tissues, one consistent feature of the miRNA expression pattern was the striking up-regulation of miR-21 in all three tumors compared with its expression in

nonneoplastic controls ($P < 0.05$). Several additional miRNA candidates differentially expressed by the arrays (Supplemental Data 2) have not been validated as significantly dysregulated in the Northern blot analysis of multiple glioblastoma multiforme samples and/or derived cell lines.

We confirmed the array-identified overexpression of miR-21 in gliomas by Northern blot using a variety of mammalian tissues and cells (Fig. 1B). miR-21 was strongly elevated in all high-grade glioma samples tested, including glioblastoma multiforme tumor tissues from five patients as well as the 12 early-passage cultures established from four additional patients. Its expression was increased 5- to 100-fold in human glioblastoma multiforme tissue compared with control nonneoplastic brain. We found a similarly robust increase in miR-21 expression in six commonly used model cell lines derived from human glioblastomas (U373, A172, LN229, U87, LN428, and LN308; Fig. 1C). In most cases, the concomitant up-regulation of the 72-nucleotide miRNA precursor was also apparent on the Northern blots. The increased expression in the human glioblastoma multiforme cell lines ranged from 5- to 30-fold compared with controls. Controls included nonneoplastic adult human and adult mouse brain tissues (cortex and white matter), fetal human and mouse brain tissue at multiple stages of development, rat primary neurons and astrocytes, and rat embryonic and adult stem cell-derived neural precursors, neurons and glia. All of these controls showed either trace or no expression of miR-21 compared with glioblastoma samples. In contrast to glioblastomas, a limited sampling of anaplastic astrocytoma, oligoastrocytoma, oligodendroglioma, and medulloblastoma suggests that some increase in miR-21 expression is present in other primary brain tumors but that very high levels of this miRNA are more typical of glioblastomas (Fig. 1D). We hypothesized that aberrant increased expression of this miRNA might block the expression of gene products that promote normal glial differentiation or that induce apoptosis, holding tumor cells in an inappropriately primitive and proliferative developmental state.

To study the biological significance of miR-21-elevated expression, we used a loss-of-function approach in glioma cell lines. We employed the U87, A172, LN229, and LN308 human glioblastoma cell lines as these representative lines have genetic abnormalities common in glioblastoma (17), show rapid growth in culture, and have strongly elevated expression of miR-21 (Fig. 1C). In addition, these cell lines are readily transfectable with small synthetic oligonucleotides in small lipid micelles, with 90% to 100% of cells showing uptake of a fluorescent-tagged marker oligonucleotide at 8 hours post-transfection (data not shown). For miR-21 suppression, we employed two similar strategies, both of which use chemically synthesized modified oligonucleotides. Recently, a strategy was reported that uses 2'-*O*-methyl-oligoribonucleotides as sequence-specific inhibitors of miRNA function and miRNA-directed RISC activity (18, 19). These molecules stoichiometrically bind and irreversibly inactivate miRNAs, providing a valuable tool to disrupt the function of a single miRNA *in vitro* and *in vivo*. We transfected different concentrations of the 2'-*O*-methyl-oligonucleotide complementary to miR-21 into the cell lines and the cells were analyzed by Northern blots 2 days post-transfection. These analyses confirmed that the target miRNA became undetectable after introduction of the 2'-*O*-methyl-oligonucleotide in the low-nanomolar range (Fig. 2), likely due to the formation of highly stable complexes with the blocking oligonucleotide that prevents miRNA detection even under the strong denaturing conditions used in the Northern blot. The effect

was sequence-specific; miR-21 was blocked by the corresponding antisense 2'-*O*-methyl-oligonucleotide (2'OMe-miR-21) but not by a scrambled or unrelated 2'-*O*-methyl-oligonucleotide (2'OMe-EGFP). Other miRNAs tested were not affected by 2'-*O*-methyl-oligonucleotide complementary to the miR-21.



We also employed an alternative technique to inhibit miRNA function based on LNA/DNA-mixed antisense oligonucleotides. In principal, it is similar to the 2'-*O*-methyl-oligonucleotide approach; antisense oligonucleotides containing LNAs form highly stable duplexes with high specificity for complementary RNAs (20, 21) and when bound to a miRNA prevent miRNA interaction with the RISC protein complex and with an mRNA target.⁵ The extremely high-affinity binding by LNAs can be exploited for superior recognition of an intracellular target. Mixed LNA/DNA oligonucleotides with eight LNAs located either at the center or at the extremities of the sequence can be used for miRNA inhibition. We used this approach in parallel with the 2'-*O*-methyl-oligonucleotides to inhibit miR-21 and found them similarly efficient and specific in the same range of concentrations when transfected with the same type of cationic liposomes (Fig. 2; "LNA-miR21" compared with its control "scrambled" oligonucleotide).

In determining the biological effects of miR-21 inhibition, we focused on two fundamental characteristics of neoplastic cells: the propensity of tumor cells to proliferate and their resistance to apoptosis. We detected a significant drop in cell number in cultures transfected with either 2'-*O*-methyl-miR-21 or LNA/DNA-miR-21, but not with unrelated 2'-*O*-methyl- or LNA/DNA-oligonucleotides (Fig. 3A). The metabolic activity of these cultures, reflecting the number of viable cells, was severely reduced with suppression of miR-21 but not with the control oligonucleotides or with suppression of two other miRNAs (miR-124a and miR-125b) that are abundant in normal and tumor brain, and this effect was seen across multiple cell lines (Fig. 3B). Repeated targeting of miR-21 in cell culture by transfections of LNA/DNA oligonucleotides further reduced the cell number. However, the cell number reduction was more modest in cells previously treated with LNA-miR21 compared with the effects on naive glioblastoma cells (Fig. 3C). Presumably, the subsequent diminished effect is due to increasing selection of resistant cells. The effect of the second transfection on remaining cells could indicate that either some cells were not efficiently transfected in the previous treatment or that some surviving cells could reexpress miR-21 and be vulnerable to later targeting.

The change in cell number was not due to large differences in proliferation as measured by bromodeoxyuridine incorporation (data not shown); rather, the decrease in cell number was associated instead with a marked increase in apoptosis (Fig. 4). Caspase-3 and caspase-7 enzymatic activities (key executioners of apoptosis) increased 3-fold by 48 hours post-transfection in cells treated with the antisense-modified oligonucleotides relative to

⁵ I. Naguibneva et al., "MicroRNAs in terminal muscle differentiation," abstract in "siRNAs and miRNAs" 2004 Keystone Symposium and article submitted for publication.

Figure 4. Suppression of miR-21 results in caspase activation and increased apoptosis. **A**, caspase-3 and caspase-7 activation after transfection with the indicated oligonucleotides. **B**, quantification of % apoptotic nuclei visualized by TUNEL staining after transfection with the indicated oligonucleotides. **C**, representative images of apoptotic nuclear TUNEL staining visualized with Cy3 (red) at 20 \times magnification. 4',6-Diamidino-2-phenylindole counterstain (blue) highlights all nuclei. Transfection with 2'OMe-miR21 and LNA-miR21 increased apoptosis compared with the control oligonucleotides, 2'OMe-EGFP and LNA-scrambled. LNA-miR-21 also caused focal lifting of cells (two representative panels shown). **A**, columns, mean (calculated from three separate experiments of five replicates each); bars, SE. **B**, bars, SD. $P < 0.01$ (z test) for all comparisons of LNA-miR-21 and 2'OMe-miR-21 with their respective controls in (A) and (B).

mock-transfected cells or cells treated with the control oligonucleotides (Fig. 4A). In addition, fluorescent TUNEL staining indicated a significant increase in the percent of fragmented DNA and apoptotic nuclei in the miR-21-blocked cells at 48 hours post-transfection (Fig. 4B and C). Treatment with LNA-miR21 was also noted to cause lifting of dead cells.

Our data therefore indicate that miR-21 acts as an antiapoptotic factor in glioblastoma cells. We propose that aberrantly increased expression of miR-21 may down-regulate the translation or stability of mRNA(s) coding for apoptosis-related genes. The precise targets of miR-21 remain elusive at present, as is the case with virtually all the known mammalian miRNAs. However, with increasingly sophisticated algorithms for target prediction and techniques for mRNA expression and proteome analysis, identification, and validation of miRNA targets including those for miR-21 will be accomplished in the near future.

Expression of miR-21 is not unique to glioblastoma. Indeed, our own data show that a low level of expression is present in normal white matter in the mature brain and in a variety of other primary brain tumors. Other adult human tissues likely express miR-21 to some extent as well, as is the case in a variety of murine tissues. In the mouse, miR-21 can be detected in liver, heart, ovary, kidney, and lung at levels ranging from 1.5- to 2-fold higher than that found in the brain (22). In comparison, however, the 5- to 100-fold change in expression we have found in glioblastoma samples versus nonneoplastic brain is striking. Interestingly, miR-21 and its precursor also have been reported to be strongly expressed in cell lines established from colorectal carcinoma (HCT-116) and cervical adenocarcinoma (HeLa) but are relatively weakly expressed in cell lines from promyelocytic leukemia (HL-60), chronic myelogenous leukemia (K562), and prostatic adenocarcinoma (LNCAP; ref. 23). A recent unpublished technical note from Ambion (November 2004) has suggested that miR-21 is overexpressed in some other tumor types as well. Our analysis of colon, kidney, and breast tumor RNA, although limited for very few samples, suggests a more modest up-regulation of miR-21 expression in these tumors

compared with glioblastoma multiformes (Fig. 1B; Supplementary Data 3). Interestingly, a recently published article by Cheng et al. reported that several miRNAs, including miR-21, can regulate cell growth and apoptosis in HeLa cells (24). However, in contrast to the effect in glioblastoma cells that we report here, miR-21 suppression increased growth of HeLa cells without affecting their apoptosis. In other cells lines tested, however, such a growth effect was not detected. The different biological effects of any particular miRNA, including miR-21, in different cells are likely to be highly dependent on the cell-specific repertoire of target genes. Thus, a high level of miR-21 expression and perhaps its antiapoptotic activity may seem common for some but not all types of tumors.

In summary, we have found that miR-21 is commonly and markedly up-regulated in human glioblastoma and that inhibiting miR-21 expression leads to caspase activation and associated apoptotic cell death in multiple glioblastoma cell lines. These findings suggest that overexpressed miR-21 may function as a micro-oncogene in glioblastomas by blocking expression of key apoptosis-enabling genes. To our knowledge, the presented work is the first to ascribe a function to an aberrantly expressed miRNA in human neoplasia. Moreover, it is now clear that miRNAs cannot be overlooked as an entire class of molecules critical to the biology and possibly to the treatment of human malignancies.

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