miR-21 upregulation and miR-128a downregulation in human glioblastomas

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INTRODUCTION

The human glioblastoma multiforme (GBM) represents the most common and lethal type of brain tumor, due to high invasive capacity and difficult early diagnosis. Several factors, including the selective blood-brain barrier and tumor cell resistance to chemotherapy, contribute to lack of efficacy of the existing therapies. Therefore, there is an urgent need to explore new treatment options for brain tumors. The discovery of microRNAs (miRNAs) has revealed an additional level of gene regulation with important consequences to the modulation of cell fate. miRNA deregulation has been reported for most human cancers and associated with progression and development of this disease. Several miRNAs, including miR-128a and miR-21, were found to be implicated in the modulation of glioma oncogenesis. Since miRNA deregulation is an important hallmark on the oncogenic process, the goal of this work was to evaluate the levels of miR-128a, miR-21 and miR-221 in human GBM samples and cell lines, while assessing whether miRNA modulation by oligonucleotides could affect tumor cell viability.
This work demonstrates that miR-128a and miR-21 are deregulated in human glioblastoma samples and the modulation of these miRNAs with oligonucleotides decreases tumor cell viability. Moreover, this work suggests that these small molecules constitute highly promising targets for anti-tumoral strategies.

MATERIALS & METHODS

- Total RNA was extracted from cells and tissues and enriched for miRNAs using the mirVana miRNA isolation kit (Ambion). Real-time PCR (qPCR) was performed using miRNA-specific primer sets, reagents and protocols from Applied Biosystems in a 7300 Real-Time PCR system (Applied Biosystems). miRNA input was normalized to the level of the housekeeping U6snRNA. Relative quantification of miRNA expression was calculated using the ΔΔCt method.
- Transfections were performed using the LipofectAmine RNAiMax reagent (Invitrogen) following the manufacturer's procedure.
- One Way ANOVA analysis of variance combined with Tukey posthoc test was used for multiple comparisons. Data analysis was performed with PRISM 4.0 (GraphPad, San Diego, CA, USA).

RESULTS

1. miR-21 upregulation and miR-128a downregulation in MGPP1 mouse cell line

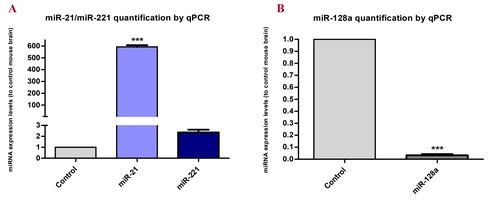

Figure 1 – qPCR quantification of miR-128a, miR-21 and miR-221 in MGPP1 mouse glioma cell line*. This cell line was established by culturing cells from a tumor of a mouse injected with PDGF-IRES-CRE expressing retrovirus. *PTEN$^{(-/-)}$P53$^{(-/-)}$PDGF$^{(+/+)}$. (**A**) miR-21 is upregulated in MGPP1 cells compared to normal mouse brain (p<0.001), with a mean value of 592.3 ± 35.14. miR-221 is also upregulated, with a mean value of 2.357 ± 0.6114, without reaching statistical significance (p>0.05). (**B**) miR-128a is downregulated in MGPP1 cells compared to normal mouse brain (p<0.001), with a mean value of 0.033 ± 0.02223. *** p<0.001 compared to control normal mouse brain.

RESULTS

2. miR-21 upregulation and miR-128a downregulation in U87 human glioblastoma cell line

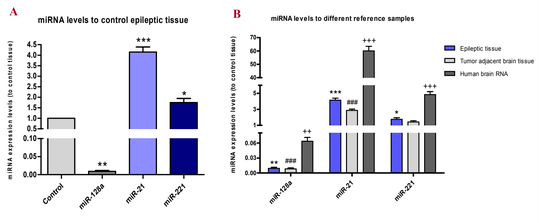

Figure 2 – qPCR quantification of miR-128a, miR-21 and miR-221 in U87 human glioblastoma cell line. (**A**) miR-21 is upregulated in U87 cells compared to epileptic tissue (p<0.001), with a mean value of 4.145 ± 0.4927, whereas miR-221 is upregulated (p<0.05) with a mean value of 1.750 ± 0.3906. miR-128a is downregulated when compared to epileptic tissue (p<0.01), with a mean value of 0.00925 ± 0.0045. *p<0.05, **p<0.01, ***p<0.001 compared to epileptic tissue. (**B**) miRNA expression levels compared to three reference samples: epileptic tissue, brain tissue adjacent to brain tumor and commercially acquired total RNA from human brain (Clontech). ### p<0.001 compared to tumor adjacent epileptic tissue, ++ p<0.01, +++ p<0.001 compared to RNA from human brain.

3. Modulation of miR-21 and miR-128a levels in U87 cells by antimiR-21 oligonucleotides and precursors of miR-128a

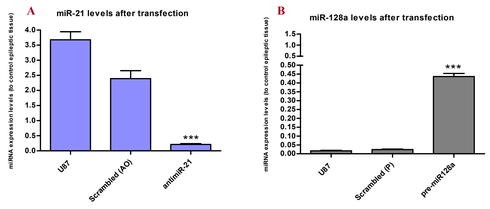

Figure 3 - qPCR quantification of miR-128a and miR-21 in U87 cells after cell transfection with antimiR-21 oligonucleotides or miR-128a precursors, at a final concentration of 80nM. (**A**) 10-fold reduction in miR-21 levels after transfection with antimiR-21 oligonucleotides, when compared to a scrambled sequence (p<0.001). (**B**) 18-fold increase in miR-128a levels after transfection with miR-128a precursors, when compared to a scrambled sequence (p<0.001). ***p<0.001 compared to a scrambled sequence.

4. Cell viability (U87) after transfection with antimiR-21 oligonucleotides and precursors of miR-128a

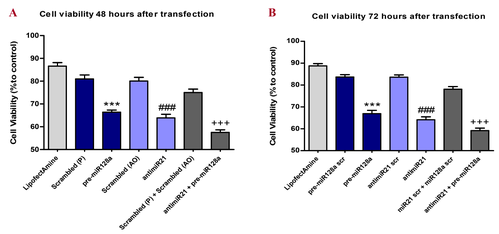

Figure 4 – Cell viability (MTT assay) after transfection with antimiR-21 oligonucleotides and/or miR-128a precursors, at a final concentration of 80nM. (**A**) Transfection with antimiR-21 oligonucleotides or miR-128a precursors resulted in 15% reduction on cell viability after 48 hours (p<0.001). A synergistic effect (20% reduction on cell viability) is observed when both molecules are transfected simultaneously (p<0.001). (**B**) A similar synergistic effect (25% reduction on cell viability) is observed 72 hours after transfection (p<0.001). ***p<0.001, ###p<0.001, +++p<0.001 to the respective control scrambled sequence.

RESULTS

5. miR-21 downregulation in PDGF-overexpressing U87 cells

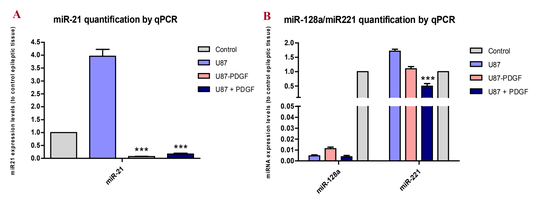

Figure 5 – qPCR quantification of miR-128a, miR-21 and miR-221 in a PDGF-overexpressing U87 cell line (U87-PDGF)*. This cell line was established by injecting PDGF-IRES-CRE expressing retrovirus in regular U87 cells. * PDGF$^{(+/+)}$ (**A**) miR-21 is downregulated in U87-PDGF cells when compared to regular U87 cells and epileptic tissue (p<0.001), with a mean value of 0.068 ± 0.02387. Moreover, a similar downregulation value is achieved when growing U87 cells in medium containing 30 ng/mL PDGF. ***p<0.001 compared to regular U87 cells. (**B**) miR-128a and miR-221 levels are not significantly different between U87-PDGF cells and regular U87 cells. ***p<0.001 compared to regular U87 cells.

6. Modulation of miR-21 and miR-128a levels in U87-PDGF cells by antimiR-21 oligonucleotides and precursors of miR-128a

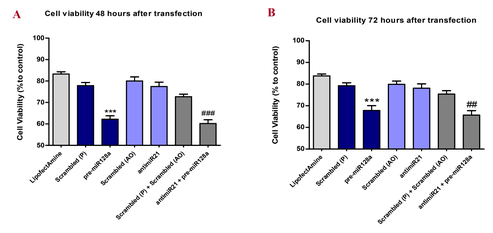

Figure 6 – Cell viability (MTT assay) after transfection with antimiR-21 oligonucleotides and/or miR-128a precursors, at a final concentration of 80nM. (**A**) Transfection with antimiR-21 oligonucleotides had no significant effect on cell viability (p>0.05) whereas transfection with miR-128a precursors resulted in 15% reduction on cell viability (p<0.001). No synergistic effect is observed when both molecules are transfected simultaneously (p<0.001). (**B**) A similar effect is observed 72 hours after transfection (p<0.001). ***p<0.001, ##p<0.01 to the respective control scrambled sequence.

7. miR-21 upregulation and miR-128a downregulation in human glioblastoma samples

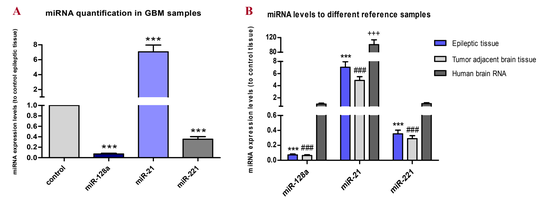

Figure 7 – qPCR quantification of miR-128a, miR-21 and miR-221 in 22 human glioblastoma samples. (**A**) miR-21 is upregulated in 80% of the tumor samples when compared to epileptic tissue (p<0.001), with a mean value of 7.047 ± 6.068. miR-221 is downregulated in 91% of the samples (p<0.001), with a mean value of 0.3536 ± 0.3288, whereas miR-128a is downregulated in all tumor samples (p<0.001), with a mean value of 0.0721 ± 0.0839. *** p<0.001 compared to epileptic tissue. (**B**) miRNA expression levels compared to three reference samples: epileptic tissue, brain tissue adjacent to brain tumor and commercially acquired total RNA from human brain (Clontech). ### p<0.001 compared to tumor adjacent epileptic tissue, +++ p<0.001 compared to RNA from human brain.

RESULTS

8. miR-21 overexpression and miR-128a underexpression in human GBM samples from The Cancer Genome Atlas

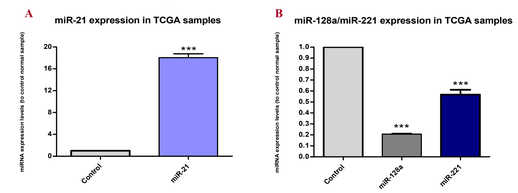

Figure 8 – Microarray analysis of miR-128a, miR-21 and miR-221 in 252 human samples (The Cancer Genome Atlas Network, 2008). An Agilent 8x15K human miRNA-specific microarray platform was used for miRNA expression analysis. (**A**) miR-21 is significantly upregulated in all tumor samples when compared to control normal samples (p<0.001), with a mean value of 18.04 ± 9.565. *** p<0.001 compared to control normal samples. (**B**) miR-128a is downregulated in all tumor samples (p<0.001), with a mean value of 0.2067 ± 0.1011 whereas miR-221 is deregulated in 72% of the samples (p<0.001), with a mean relative value of 0.57 (0.5672 ± 0.5946). This miRNA is downregulated in 93.5 % of the samples. *** p<0.001 compared to control normal normal samples.

CONCLUSIONS

- miR-21 is upregulated and miR-128a is highly downregulated in human glioblastomas. Analysis of the Cancer Genome Atlas Research Network data on 252 human glioblastomas corroborates our experimental data.
- miR-221 is deregulated in human glioblastomas. Upregulation and downregulation of this miRNA are observed among tested samples.
- Silencing miR-21 or overexpressing miR-128a with antisense oligonucleotides or precursor molecules successfully decreases tumor cell viability. A synergistic effect is achieved when the two strategies are conjugated.
- Decrease in tumor cell viability is proportional to the levels of the miRNAs in tumor cells. In a cell line with low levels of miR-21, the silencing of this miRNA does not affect significantly cell viability.
- miRNA-targeting approaches can provide an alternative/complementary therapy in tumors where the miRNA milieu is deregulated.

FUTURE DIRECTIONS

- Identify the mechanisms by which miR-128a and miR-21 are deregulated in human glioblastomas.
- Clarify the interaction between PDGF and miRNAs.
- Develop and test delivery vehicles that reliably and effectively overcome cellular and/or physiological barriers, and allow for specific delivery of the oligonucleotides to the tumor.

ACKNOWLEDGEMENTS

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