

Cell type-specific Effect of miRZip-21 to Suppress miR-21 in Human Glioma Cell Lines

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Abstract

There are different subtypes of brain tumors, classified according to the origin of the abnormally proliferated glial cells. Glioblastoma multiforma (GBM) is the grade 4 of brain tumors, gliomas, with the least life expectancy. microRNAs (miRNAs) are small, single stranded, non-coding RNAs with 20-25 nt length with post-transcriptional gene regulatory activities. An altered expression of miRNAs is linked to developmental disorders and some diseases, most importantly cancers. miR-21 is a well-known microRNA, overexpressed in almost all cancer types, including brain tumors. It targets several genes with vital roles in cellular pathways involve in proliferation, invasion and metastatic behaviors. Exosomes are 30-100 nm extracellular vesicles which are packed with various molecules, including miRNAs. Here, we suppressed miR-21 expression level in HEK-293T cells by transfecting them with the miRZip-21 vector. However, when U87-MG cells were cultured in the presence of exosomes isolated from conditioned medium of engineered HEK-293T cells derived exosomes, we did not observe any suppressing effect on host cells' miR-21 expression level. Moreover, by analyzing the effects of miRZip-21-enriched cell's conditioned media on three other brain cell lines including 1321N1, A-172 and DAOY, cell type-specific effects of exocrine miRZip-21 were revealed. These data suggested that cell lines from different brain tumor subtypes could exert different responses to microRNA-based therapies, based on their cellular origin and clinical behaviors.

Keywords: miR-21, Brain tumors, Glioblastoma multiforma, Exosomes

Introduction

Brain tumor is a neoplasm that occurs by an abnormal and uncontrolled cell division of glial cells within the central nervous system (CNS). Tumors of the CNS have a wide spectrum of subtypes according to the WHO classification, their cellular origin and histological characteristics of each particular tumor. The most frequent brain cancers in children are pilocytic astrocytomas, ependymomas and medulloblastomas, while, in adults diffuse astrocytic tumors (including astrocytoma, anaplastic astrocytomas, and glioblastomas), oligodendrogliomas, and meningiomas (Collins and Psychiatry, 2004; Louis et al., 2007; Louis et al., 2016) are most frequent brain cancer subtypes. Glioblastoma multiforma (GBM) is the grade 4 of brain tumors, which its life expectancy about 18 months after diagnosis (Paolillo et al., 2018).

microRNAs (miRNAs) are small, single stranded, non-coding RNAs with 20-25 nt length that have post-transcriptional gene regulatory

activities. miRNAs play crucial roles in variable biological events such as development, differentiation and apoptosis. Misregulations in expression level of miRNAs can lead to some diseases including cancers. Therefore, investigating the expression profile of various miRNAs can provide useful diagnostic, prognostic and therapeutic information for the future therapeutic challenges (Gulyaeva and Kushlinskiy, 2016; Hayes et al., 2014; Paul et al., 2018). miR-21 is a well-known miRNA that is overexpressed in almost all cancer types, including brain tumors. It targets several important genes in cellular processes, with regulatory effects on proliferation, invasion and metastatic behaviors. PDCD4 (Programmed cell death protein 4) and RECK (Reversion-inducing-cysteine-rich protein with kazal motifs) are two important miR-21 target genes, with some key regulatory roles in apoptotic and metastatic pathways (Corsten et al., 2007; Gabriely et al., 2008; Gao et al., 2007; Gaur et al., 2011; Malhotra et al., 2018; Papagiannakopoulos et al., 2008; Sekar et al., 2015). Inhibition of miR-21 expression or activity via different techniques promoted apoptotic cell death, sensitivity to chemotherapy/radiotherapy and cancer suppression (Belter et al., 2016;

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Devulapally et al., 2015; Lu et al., 2008; Sicard et al., 2013; Yang et al., 2014).

Extracellular vesicles (EVs) are membrane fragments budding from cells' surfaces to transfer cytoplasmic or membrane cargoes to target cells (Ratajczak et al., 2006; Tkach and Théry, 2016; Yuana et al., 2013). Exosomes are 30-100 nm extracellular vesicles with important roles in signaling pathways (Arscott et al., 2013; Kucharzewska et al., 2013; Mittelbrunn et al., 2011). They contain a variety of cellular components, including proteins and genetic materials such as miRNAs (Nouraei et al., 2015; Yu et al., 2016; Zhang et al., 2015).

Here, we tried to downregulate miR-21 expression in HEK293T cells by transfecting them with an anti-miR-21 (miRZip-21) construct. Then, engineered exosomes enriched from HEK293T cells' conditioned media were transferred into glioblastoma cells to explore their suppressing effects on host cells' miR-21 expression level. Our data revealed that exosome enriched with miRZip-21 have differential effects on different glioma cell lines.

Materials and Methods

Cell culture and transfection

HEK-293T cells were obtained from Iranian biological resource center and cultured in DMEM-F12 (Gibco, USA), supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Bio Basic, Canada) and were seeded in a 12-well plate (SPL Life Science, South Korea). To suppress miR-21 expression level, miRZip-21 purchased from System Bioscience (SBI, USA). Stable cell line colonies expressing miRZip-21 were generated by transfecting cells at %70 confluencies with lipofectamin 3000 (Invitrogen, USA). Successfully transfected cells were selected by using 4 µg/ml Puromycin (Sigma, Germany). Then, cells were expanded while antibiotic concentration reduced gradually.

RNA extraction and RT-PCR

RNA extraction performed by Trizol (for cells) and Trizol LS (for cell's conditioned media and exosomes) reagents (Invitrogen, USA). After cDNA synthesis with TAKARA cDNA synthesis kit (Japan), Real-time PCR was performed for detecting miR-21 expression level using SYBR Green (Bio Fact, Korea) and Stem-loop method (Kramer, 2011). Then, the expression levels of PDCD4 and RECK, as miR-21 target genes, were analyzed (Supplementary table 1).

Co-culture experiments

In order to analyze miRZip-21 effects on target cells, stable transfected HEK-293T cells were seeded in 6-well plates (2×10⁴ cells per well, SPL Life Science, South Korea). 24 hours later, target cells (U87-MG cells, obtained from Iranian biological resource center) were seeded on inserts with 0.4 µm pore sizes (SPL Life Science, South Korea), within the same plates of transfected HEK-293T cells (23×10³ cells/insert). RNA extraction was performed for both HEK-293T and U87-MG cells after 24 and 48 hours of co-culture experiments.

Exosome purification and characterization

miRZip-21-expressing stable cell lines were cultured in T75 cell culture flasks in the presence of exosome-depleted FBS (Huan et al., 2013). Cell's conditioned media were collected every 2-3 days, and total exosomes were extracted by several steps of centrifugation (300 g for 10 min, 2000 g for 10 min, 10000 g for 30 min, 20000 g for 60 min, 100000 g for 70 min) (Théry et al., 2006). DLS analysis (with 10 minutes sonication), and Bradford assay were respectively used to determine size and concentration of isolated exosomes. 20 µl of our exosome preparations were applied for scanning electron microscopy (SEM). To visualize size and shapes of extracted exosomes, SEM images were achieved by using gold coating with physical vapour deposition (PVD) method, done with Sputter coater instrument (SBC 12 model) and KYKY-EM3200 instruments (26 KV).

Investigating effects of exosomes on U87-MG cells: U87-MG cells were seeded in a 24-well plate in RPMI media (Gibco, USA) (supplemented with 10% FBS and 1% Penicillin/streptomycin). Then, they exposed to 50 µg/ml of miRZip-21 enriched exosomes for 24 to 48 hours. Following cell lysis, total RNA extraction performed for all samples to investigate the expression levels of miR-21 and miR target genes.

Analyzing effects of conditioned media from miRZip-21 producing HEK-293T cells on other brain related cell lines: 1321N1, A172 and DAOY cell lines were obtained from Iranian biological resource center, cultured in RPMI media (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin (Bio Basic, Canada), in 24-well plate (SPL Life Science, South Korea). Upon reaching 70% confluencies, their medium exchanged with an equal mixture of RPMI and conditioned media of miRZip-21 producing HEK-293T cells (1:1).

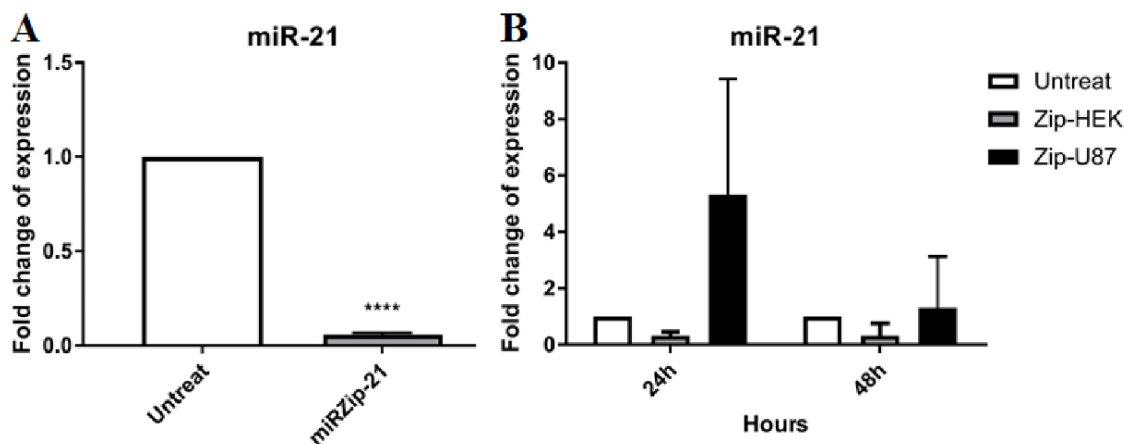


Figure 1. A) Down-regulation of miR-21 expression in HEK-293T stable cell lines exogenously expressing miRZip-21, in comparison to untransfected cells ($p < 0.0001$). B) miR-21 expression level in U87-MG cells, 24 and 48 hours following co-culturing with HEK-293T cells stably expressing miRZip-21. As demonstrated no significant downregulation observed for treated cells.

Statistical analysis

All experiments were repeated at least 2-3 times. Statistical analysis performed using GraphPad Prism 6 software and ordinary ANOVA test. Data were presented as Mean \pm SD, and differences were considered significant, when p value were less than 0.05.

Results

miRZip-21 decreases miR-21 expression level in HEK-293T cells: We employed miRZip-21 to downregulate miR-21 expression level. Following transfecting HEK-293T cells with miRZip-21 vector and producing stable cell lines, the expression levels of miRZip-21 and miR-21 were determined by a real-time PCR approach. In miRZip-21 expressing stable cell line, miR-21 expression level significantly decreased in comparison to untransfected cells ($p < 0.0001$; Figure 1A). Gene expression levels were normalized to the expression of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as our house keeping gene.

miR-21 expression level of U87-MG cells exert modifications upon co-culturing with HEK-293T cells expressing miRZip-21: Primary glioblastoma cell line U87-MG was employed to analyze the effects of secreted miRZip-21 in a co-culture system, in which the cells were physically separated from each other. After 24 and 48 hours of conditioned media contact between U87 and

miRZip-21 expressing HEK-293T cells, miR-21 expression measured quantitatively. Our experiments revealed that although, miRZip-21 decreases miR-21 expression level in HEK-293T, but it fails to decrease miR-21 level in U87-MG target cells (Figure 1B). As it is evident in Figure 1, we even observed an unexpected elevation of miR-21 levels in U87-MG target cells following 24 h and 48 h of co-culture experiments.

miRZip-21 successfully packaged into the exosomes of transfected cells: To examine the possibility of miRZip-21 packaging in the exosomes of transfected cells, we purified exosomes by means of ultracentrifugation. General characteristics of isolated exosomes including their size and shape evaluated and confirmed by performing DLS analysis and electron microscopy. Particle size analysis revealed a sharp and single peak on 83.46 nm point for purified vesicles (Figure 2A). Uniform shape and proper size of isolated exosomes (mostly under 100 nm) were also confirmed following scanning electron microscopy experiments (Figure 2B).

We then analyzed the level of miR-21 expression in exosomes isolated from conditioned media of HEK-293T miRZip-21 expressing stable cell line, in comparison to untransfected cells. Our data revealed a diminished level of miR-21 in the miRZip-21 expressing cells; however, it was not statistically significant (Figure 3).

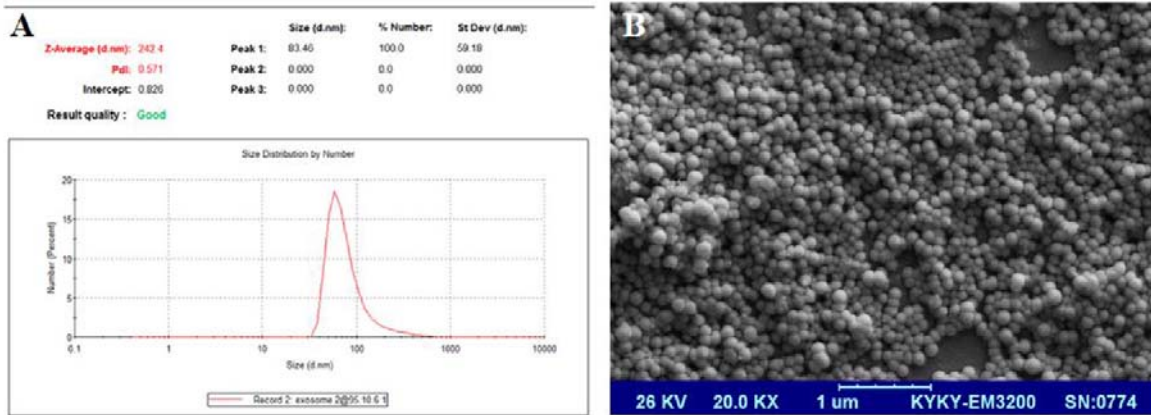


Figure 2. Characterizing the purity and identity of extracted exosomes. A) DLS experiments demonstrated a good quality and normal size distribution (with a unique peak under 100 nm; average 83.46 nm) for purified exosomes. B) SEM micrograph obtained from our exosome preparation, indicated proper concentration of exosomes in our investigated sample with ideal size distribution between 30-170 nm (mostly under 100nm).

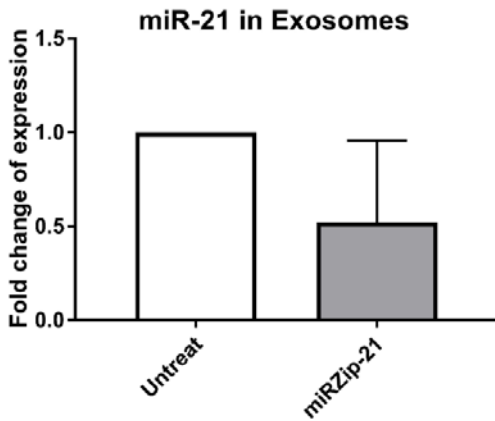


Figure 3. miR-21 expression levels in exosomes isolated from miRZip-21 expressing HEK-293T cells, in comparison to the exosomes extracted from untransfected cells.

Engineered exosomes had no miR-21 suppression effect on U87-MG cells: Despite our expectations, miRZip-21 containing exosomes failed to exert any suppression effects on miR-21 expression levels of U87-MG cells as our target cell (Figure 4A). Interestingly, a noticeable, but not statistically significant, increase in the level of miR-21 was observed in the cells, 24h after co-culture experiments. To explore the possibility of a similar effect of miRZip-21 containing exosomes on miR-21 target genes, we quantified the expression levels of PDCD4 and RECK genes, two important target genes of miR-21, in treated cells. As was expected, miRZip-21 containing exosomal treatments caused a reverse effect on the expression of PDCD4 and RECK genes in treated cells, although, these changes were also not statistically significant (Figure 4B, C).

Investigating miRZip-21 effects on other brain cancer cell lines: To further examine the effects of miRZip-21 on other cell lines, we performed similar experiments on some other glioma cell lines (table 1). Our data revealed a differential effect on the above mentioned cell lines exposed to the miRZip-21-enriched conditioned media of HEK-293T stable cells (Fig 5). In 1321N1 astrocytoma cell line, miR-21 level elevated in 24 hours, but dramatically dropped down after 48 hours of treatment. In case of A172 cells, which are classified as non-tumorigenic glioblastoma cell line, a significant downregulation of miR-21 observed in all investigated time points. Finally, DAOY cells showed an upregulation of miR-21 expression at both 24 and 48 hours. However, differences were not statistically significant.

Discussion

Glioblastoma multiforma is the most malignant form of brain tumors (Collins and Psychiatry, 2004; Louis et al., 2007; Louis et al., 2016). Finding a new therapeutic approach for treating brain tumors is of great importance. Despite new molecular and surgical approaches (Gilbert, 2011; Van Meir et al., 2010), finding an effective cure is still remained to be introduced. microRNAs are demonstrated to have important roles in cancer initiation and progression (Gulyaeva and Kushlinskiy, 2016; Hayes et al., 2014; Paul et al., 2018). We have already reported that miR-21 is upregulated in esophageal tumors and that the upregulation was mainly confined to the fibroblast-like stromal cells adjacent to cancer cells, rather than tumor cells (Nouraei et al., 2013). The latter finding suggests a role for secreted miR-21 as a microenvironmental communication signal between

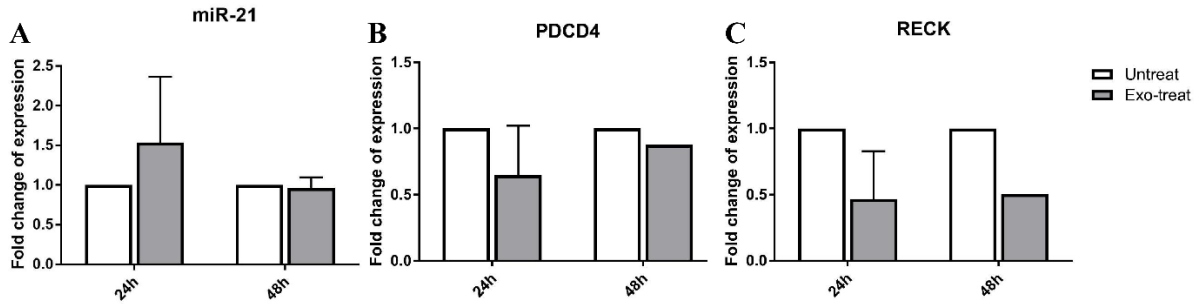


Figure 4: A) Expression of miR-21 in U87-MG cells exposed to the engineered exosomes isolated from conditioned media of miRZip-21 expressing HEK-293T cells by ultracentrifugation. B, C) Expression of PDCD4 and RECK in U87-MG cells following treatment with exosomes obtained from conditioned media of genetically modified HEK293T cells. As demonstrated similar expression pattern observed for miR-21 target genes, PDCD4 and RECK, 24 and 48 hours post-treatments. It should be notified that these modifications were not statistically significant.

Table 1: Characteristics of brain tumor cell lines applied in the present study.

Investigated Cell lines	Properties	Tumorigenic?
U87-MG	Human primary glioblastoma	Yes
1321N1	astrocytoma	?
A172	glioblastoma	No
DAOY	Desmoplastic cerebellar medulloblastoma	Yes

*Data submitted from ATCC site

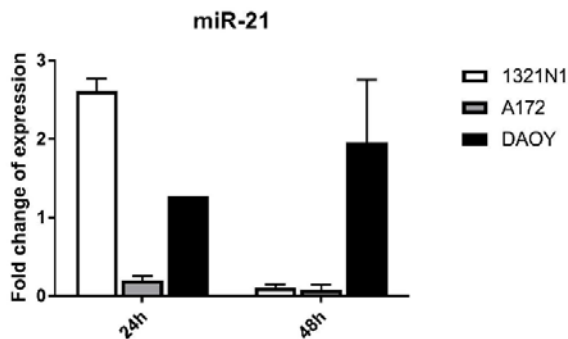


Figure 5: miR-21 expression levels in 1321N1, A172 and DAOY cell lines after treatment with conditioned media obtained from miRZip-21 stably expressing HEK-293T cells. Note that different cell lines responded differentially to the treatment with miRZip-21-containing conditioned media.

cancer cells and their adjacent non-tumor cells. Cancer cell communications is an important target point to be considered for inhibition of cancer development. Most of these secretory signaling molecules are released in the form of exosomes. GBM cells apply these microvesicles to increase tumor cell communication for proliferation, invasion and even metastatic behaviors (Arscott et al., 2013; Kucharzewska et al., 2013; Mittelbrunn et al., 2011). The presence of microRNAs within exosomes have been already reported (Collino et al., 2010; Ogata-Kawata et al., 2014; Yuana et al., 2013), including the packaging and release of miR-

21 within exosomes of different cancer cell types (Tanaka et al., 2013; Tian et al., 2014; Tsukamoto et al., 2017; Wang et al., 2015). miR-21 silencing was also carried out with effective results in both *in vitro* and *in vivo* trials (Belter et al., 2016; Devulapally et al., 2015; Lu et al., 2008; Sicard et al., 2013; Yang et al., 2014).

Here, we tried to suppress miR-21 expression in glioblastoma cell line U87-MG, employing engineered exosomes carrying the miRZip-21 construct. While miRZip-21 downregulated miR-21 expression level in transfected HEK-293T stable cell line, its effect on target cells treated with miRZip-21 containing exosomes was unexpected. There is no rational explanation for elevated expression of miR-21 in U87-MG cells. To find out if the aforementioned effect is caused by a general mechanism, we examined the effect of miRZip-21 producing stable HEK-293T conditioned media on three other brain cancer cell lines. The cerebellar medulloblastoma cell line, DAOY, showed almost similar results as U87-MG, with an upregulation of miR-21 after 24h and 48h of treatment. The effect of miRZip-21 on A172 cells was in contrast with what observed on U87-MG, with a downregulation of miR-21 expression level at both 24 and 48h of treatment. The astrocytoma cell line 1321N1 (Gundemir et al., 2017; Toll et al., 2011) showed a surprising response after treatment with miRZip-21 containing conditioned media, with an upregulation of miR-21 at 24h and a dramatic downregulation 48

hours following treatment.

Altogether, our data revealed different response of various brain cell lines to a single treatment. This is in agreement with previous findings that brain tumor subtypes response differently to different treatment approaches (Gundemir et al., 2017; Toll et al., 2011; Verhaak et al., 2010). Therefore, the therapeutic application of miR-21 suppression requires to determine the subclass of tumors, as the current proposed therapy is cell-line dependent.

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Conflict of Interest

No competing financial interests exist.

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Table S1. Primers sequence

Name	Forward / Reverse	Sequence
miR-21 cDNA Synthesis	-	5'- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACT CAA CA -3'
miR-21 PCR	Forward	5'- CCG GCC TAG CTT ATC AGA CTG -3'
	Reverse	5'- AGTG CAG GGT CCG AGG TA -3'
5S rRNA	Forward	5'- GTCTACGGCCATACCACCCTG -3'
	Reverse	5'- AAAGCCTACAGCACCCGGTAT -3'
PDCD4	Forward	5'- CAGTTGGTGGCCAGTTTATTG -3'
	Reverse	5'- AGAAGCACGGTAGCCTTATCCA -3'
RECK	Forward	5'- GACTCTTCTCCIGGTCCAATC -3'
	Reverse	5'- CTATCCGTGGGTCCICAT -3'
GAPDH	Forward	5'- ATGGGAAGTGAAGGTCG -3'
	Reverse	5'- GGGGTCATTGATGGCAACAATA -3'