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Original Research Article

MicroRNA-542 suppressed the proliferation of human glioma cells by targeting talin-2 (TLN2)

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Abstract

Purpose: *To investigate the effect of miR-542 in the development of human glioma.*
Methods: *The expressions of miR-542 and TLN2 in glioma cells and normal human astrocytes were determined using qRT-PCR, while MTT and colony formation assays were used to determine cell proliferation. Western blotting was used to determine protein expression.*
Results: *It was revealed that miR-542 was significantly downregulated in glioma cells. Overexpression of miR-542 inhibited the proliferation and clonogenicity of glioma cells via induction of apoptosis. The percentage of apoptotic U87 cells increased from 5.32 in control to 26.76 upon miR-542 overexpression. Moreover, TLN2 was identified as the functional regulatory target of miR542 in glioma. The expression of TLN2 was markedly upregulated in human glioma cells. However, overexpression of miR-542 suppressed TLN2 expression. Silencing of TLN2 mimicked the tumor-suppressive effects of miR-542 in glioma cells, but this effect was blocked by TLN2 over-expression.*
Conclusion: *These results suggest that miR-542 exerted glioma-suppressive effect, with TLN2 as its functional regulatory target.*

Keywords: *Glioma, Proliferation, Micro-RNA, Tumorigenesis, MiR-542, Apoptosis, Prognosis, talin-2, Oncogene*

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INTRODUCTION

Glioma is the most common neoplastic intracranial malignancy of the human nervous system, and it results in very high morbidity and mortality [1]. Glioma is associated with very poor prognosis, leading to extremely low patient survival [2]. Moreover, high recurrence of glioma and its invasive nature make it one of the diseases that impose major economic burdens on the patients and their families worldwide [3]. It has been reported that the average survival period of glioma patients with low-grade neoplasm is less than 60 months, while the average survival period for those with advanced pathological stages is below 15 months [4]. Therefore, there is a need for in-depth understanding of the pathogenesis of glioma in order to develop novel prognostic and therapeutic targets against this deadly malignancy.

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Micro-RNAs (miRs) are a novel class of non-coding RNAs which have been shown to participate in diverse biological pathways [5]. Categorized as short-length RNA transcripts ranging in size from 20 - 25 nucleotides, miRs lack protein coding ability, but they regulate the expressions of protein coding genes at post-transcriptional/translational level mostly via specific interactions with the untranslated regions of transcripts [6]. There is growing evidence showing that miRs regulate the proliferation, differentiation, apoptosis and migration of human cells [7]. Thus, dysregulation of miRs triggers the onset and development of a variety of human pathological conditions, including cancer [8]. Recent studies have demonstrated that several miRs show aberrant expression patterns in glioma and play tumor-promoting or tumor-suppressing roles in tumorigenesis [7]. MiR-542 acts as a tumor-suppressor in human malignancies e.g., liver cancer, by regulating the expression of specific molecular targets [9]. There are limited reports on the regulatory role of miR-542 in human glioma [10]. The present study was carried out to investigate the role of miR-542 in glioma cells.

EXPERIMENTAL

In vitro propagation and transfection of cell lines

Glioma cell lines (U87, U118 and M0159K) as well as normal human astrocytes were procured from the American Type Culture Collection (ATCC, VA, USA). The cell lines were propagated in DMEM (Thermo Fisher Scientific, Carlsbad, CA) containing 10 % fetal bovine serum (FBS, Gibco Inc.) in a humidified CO_2 incubator.

Synthetic miR-654 mimics and negative control (miR-NC) as well as small interfering RNAs of TLN2 (si-TLN2), TLN2 over-expression plasmid (pcDNA-TLN2) and their negative controls (si-NC and pcDNA3.1 vectors, respectively) were obtained from RiboBio Ghaungzhou, China. These constructs were transfected into U87 glioma cells using Lipofectamine 2000 (Thermo Fisher Scientific) as per the manufacturer's instructions.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

The extraction of total RNA from cell lines was done with TRIzol reagent (Thermo Fisher Scientific). Next, approximately 1000-1500 ng of total RNA was reverse-transcribed to cDNA using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative Real-time polymerase chain reaction (qRT-PCR) was carried out using Power SYBR® PCR Master mix (Thermo Fisher Scientific) on QuantStudio 5.0 Real-time PCR System (Thermo Fisher Scientific). The relative expression levels were quantified using $2^{-\Delta\Delta CT}$ method. The mRNA expression data were normalized to GADPH, while the miR-542 expression was normalized to that of U6.

MTT and clonogenic assays

The MTT assay was performed to determine the proliferation of U87 glioma cells. After transfection, U87 cells were seeded in 96-well plates at a density of 10^4 cells per well. The cells were cultured for 96 h at 37 °C. Cell proliferation was determined after every 24 h. At indicated time intervals, 20 µL MTT solution (Sigma-Aldrich Corp., Ronkonkoma, NY, USA) was added to each well, and the cells were again incubated at 37 °C for 4 h. The culture medium was replaced with 200 µl dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to dissolve the formazan crystals formed. Finally, absorbance (A) was read for each well at 570 nm in a spectrophotometer. For the clonogenic assay, 500 transfected cells were placed in each well of 6-well plates. After culturing for 15 days, the medium was removed and colonies were washed with PBS. The colonies were then fixed with 70 % ethanol and subsequently stained with 0.2 % crystal violet (Sigma-Aldrich). Then, the stained cells were photographed for analysis of the relative colony formation.

Apoptosis assays

After transfection, U87 glioma cells were plated in 12-well plates at a density of 2.5×10^4 cells per well and incubated for 24 h at 37 °C. Thereafter, the cells were fixed with 4 % paraformaldehyde and stained with double-staining mixture of acridine orange (AO) and ethidium bromide (EB). The stained cells were visualized under a fluorescent microscope (Olympus, Tokyo, Japan). Apart from AO/EB staining, cell apoptosis was determined using Cell Apoptosis Assay Kit (Life Technologies) in line with the manufacturer's instruction. The transfected U87 cells were trypsinized and PBS-washed, followed by incubation with a mixture of 15 µL Annexin V-FITC and 15 µL propidium iodide (PI). Finally, the stained cells were analyzed for apoptosis in a flow cytometer (FACSCalibur, BD Biosciences).

Bioinformatics and luciferase reporter assay

TargetScan 7.1 (http://www.targetscan.org) was used to predict the binding sites for miR-542 within the 3′-UTR of TLN2. To confirm the prediction, luciferase reporter activity assay was performed. In brief, miR-542 mimics or miR-NC and the luciferase reporter plasmid of 3'-UTR of TLN2 (WT or MUT, RiboBio) were co-transfected into U87 cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Cells were harvested after 48 h of transfection and their relative luciferase activities were measured using the Dual Luciferase Assay System (Promega).

Western blotting

Following 24 h transfection with miR-542 mimics or miR-NC, U87 glioma cells were lysed with RIPA lysis buffer (Sigma-Aldrich). The protein concentration of each lysate sample was determined with BCA method. Then, equal (45-µg) amounts of proteins were separated on 10 % SDS-PAGE and blotted onto PVDF membranes. The latter were blocked with 5 % skim milk (BD Biosciences) prior to incubation with specific primary antibodies overnight at 4 °C. Then, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The protein signals were detected using enhanced chemiluminescence (ECL) substrate kit. Relative protein expressions were calculated, with beta-actin as internal control.

Statistical analysis

The results are expressed as mean ± SD. The SPSS version 21.0 software was used for statistical analyses. Student's t-test was used for comparison between two groups. Values of $p < 0.05$ were considered as indicative of statistically significant differences between two groups.

RESULTS

MiR-542 inhibited glioma cell growth *in vitro*

Total RNA isolation was followed by qRT-PCR for analysis of the expression of miR-542 in glioma cell lines (U87, U118 and M059K) and normal human astrocytes. The results showed significant down-regulation of miR-542 in glioma cell lines, relative to the normal astrocytes (Figure 1 A). The U87 glioma cells were used for further characterization of molecular role of miR-542 owing to the fact that miR-542 was least expressed in this cell line. The MiR-542 mimic oligos were transfected into the U87 cells to induce over-expression of miR-542. The U87 cells transfected with miR542 mimics showed 9.5-fold over-expression of miR-542, when compared to the respective miR-NC transfected negative control cells (Figure 1 B). Results from MTT assay showed that U87 cells that over-expressed miR-542 exhibited significant decline in proliferation, relative to the corresponding negative control cells *in vitro* (Figure 1 C). Similarly, upregulation of miR-542 markedly decreased colony formation in U87 glioma cells (Figure 1 D). Taken together, these results suggest that miR-542 inhibited the growth of glioma cells *in vitro*.

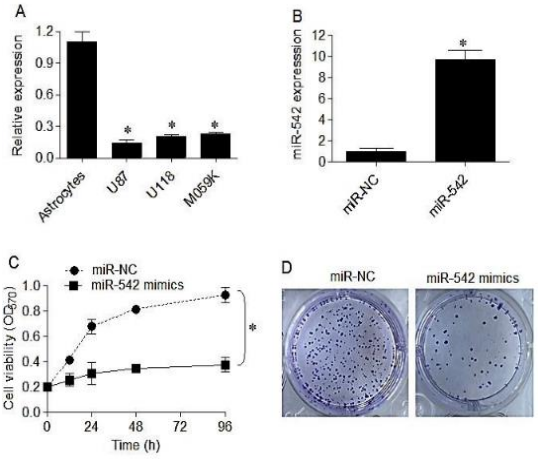

Figure 1: MiR-542 over-expression inhibited proliferation and clonogenicity of glioma cells. (A) MiR-542 was repressed in glioma cell lines (U87, U118 and M0159K), when compared to normal astrocytes. (B) Over-expression of miR-542 in U87 cells transfected with miR-542 mimics, with miRNC-transfected cells as negative control. (C) miR-542 over-expression inhibited U87 cell growth, relative to the corresponding negative control cells, as determined using MTT assay. (D) miR-542 over-expressing U87 cells exhibited significant decline in colony formation *in vitro*. *$P < 0.05$

MiR-542 re-expression induced apoptosis in glioma cells

In order to study the mechanism underlying the miR-542 over-expression-induced decrease in proliferation of U87 glioma cells, apoptosis of U87 cells over-expressing miR-542 was studied using AO/EB double staining and flow cytometry. Results from AO/EB staining showed that U87 glioma cells had higher ethidium bromide incorporation under miR542 up-regulation (Figure 2 A). Furthermore, flow cytometric analysis indicated that percentage of apoptosis of U87 cells was significantly enhanced by miR-

542 over-expression (Figure 2 B). The percentage of apoptotic U87 cells increased from 5.32 in control to 26.76 in cells with overexpression of miR-542 overexpression. Taken together, these results suggest that miR-542 induced apoptosis in U87 glioma cells.

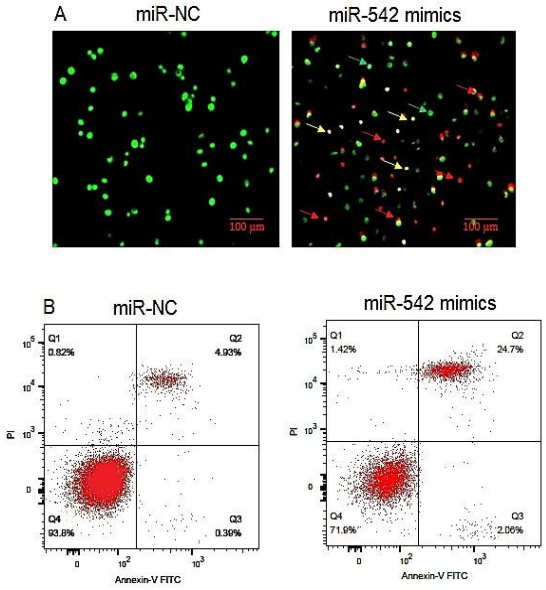

Figure 2: Over-expression of miR-542 induced apoptosis in glioma cells. (A) U87 cells overexpressing miR-542 had higher incorporation of ethidium bromide staining than negative control cells. (B) Flow cytometry showing that miR-542 up-regulation increased percentage apoptosis in U87 cells, when compared to negative control cells. Each experiment was done in triplicate

TLN2 was functional target of miR-542 in glioma

Online bioinformatics analysis was used to predict the regulatory target of miR-542. The results revealed that miR-542 targeted 3'-UTR of talin-2 (TLN2) in a sequence-specific fashion (Figure 3 A). Dual luciferase reporter assay confirmed the interaction of miR-542 with the specific binding site in TLN2 3'-UTR, as was indicated by decreases in luciferase activity of U87 cells co-transfected with miR-542 mimics and luciferase reporter plasmid of pGL3 carrying wild type (WT) miR-542 binding site (Figure 3 B). The transcript levels of TLN2 were also shown to negatively correlate with miR-542 expression in glioma cell lines (Figure 3 C). Moreover, the over-expression of miR-542 in U87 cells significantly reduced TLN2 protein expression (Figure 3 D). Silencing of TLN2 inhibited the proliferation and clonogenicity of U87 cells *in vitro* (Figure 3 E and F). These results indicate that TLN2 acted as the functional molecular target of miR-542 in glioma cells.

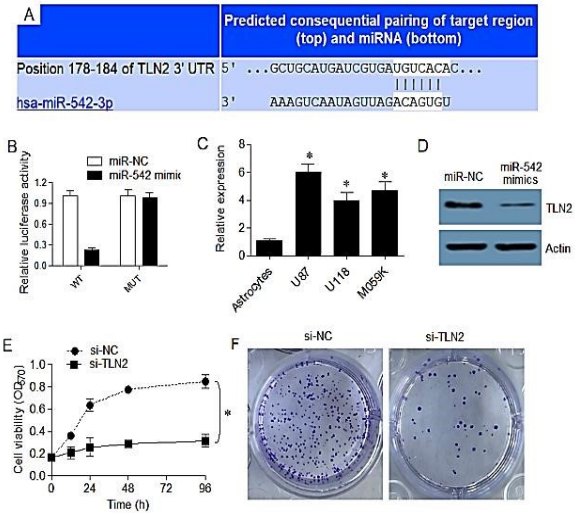

Figure 3: MiR-542 targeted TLN2 in glioma. (A) miR-542 interacted with 3'-UTR of TLN2 in a sequence-specific manner, as was determined with TargetScan analysis. (B) Dual-luciferase assay showing interaction of miR-542 with TLN2 3'-UTR. (C) TLN2 was upregulated in glioma cell lines (U87, U118 and M0159K), relative to normal astrocytes. (D) miR-542 over-expression reduced TLN2 expression in U87 cells, as was determined with western blotting. (E) TLN2 silencing inhibited the growth of U87 cells, relative to the corresponding negative control cells, as was determined using MTT assay. (D) U87 cells with TLN2 down-regulation exhibited significant decline in colony formation potential *in vitro*. *P< 0.05

Over-expression of TLN2 mitigated miR-542-induced inhibitory effect on glioma cell growth

The molecular role of miR-542 in glioma via TLN2 suppression was further confirmed by inducing TLN2 up-regulation in U87 cells over-expressing miR-542, followed by MTT assay. Results from MTT assay showed that overexpression of TLN2 attenuated the growth inhibitory effects of miR-542 up-regulation in U87 cells (Figure 4 A). Besides, clonogenic assay showed that TLN2 over-expression restored the clonogenicity of U87 glioma cells that over-expressed miR-542, with the colony formation potential being more or less equivalent to that of the negative control U87 glioma cells (Figure 4 B). These results indicate that miR-542 inhibited the proliferation of U87 glioma cells by suppressing TLN2 expression post-transcriptionally.

DISCUSSION

Right from their identification for the first time in 1993, the horizon of biological functions of micro-

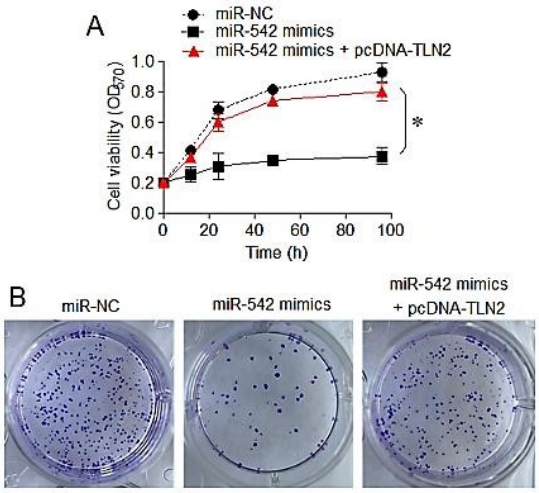

Figure 4: TLN2 over-expression mitigated anti-proliferative effects of miR-542 in glioma cells. (A) Over-expression of TLN2 restored the proliferative capacity of U87 cells. (B) TLN2 overexpression restored the clonogenicity of U87 glioma cells. **P< 0.05*

RNAs (miRs) has continued to widen [11]. Studies have shown that miRs regulate the expressions of protein-coding genes by activating their post-transcriptional degradation or suppressing their translation [12]. Research in the past 2-3 decades have revealed that miRs regulate crucial cellular processes such as proliferation, differentiation, apoptosis, migration and stress response [7]. In addition, miRs have been shown to be involved in development of diseases, especially tumorigenesis of human cancers [13]. Recent studies have reported that miRs might emerge as vital prognostic molecules and therapeutic targets against human cancers [14]. The poor prognosis, invasiveness, high recurrence and low survival of glioma patients make glioma management a very challenging task. Thus, there is need to develop more effective treatment strategies for human glioma.

In the present study, the regulatory effect of miR-542 on the progression of human glioma was investigated. The qRT-PCR analysis showed that glioma cells exhibited significantly low transcript levels of miR-542, indicating its likely involvement in regulating the malignant behaviour of host glioma cells. Previous studies have shown that down-regulation of miR-542 promoted the progression and metastasis of human cancers e.g., hepatocellular carcinoma [15]. The results of present study showed that over-expression of miR-542 induced apoptosis in glioma cells *in vitro*, thereby reducing their viability. The pro-apoptotic effect of miR-542 over-expression in neuroblastoma cells has also been reported previously [16]. In addition, it has been reported that induction of mitotic arrest mediated the anti-proliferative effects of miR542 against human cancer cells [17]. Bioinformatics was used to identify the specific molecular target of miR-542 in glioma, and the results showed that miR-542 targeted talin 2 (TLN2). This was confirmed using dual luciferase reporter assay and expression studies. Knock-down and over-expression of TLN2 in glioma cells confirmed that it (TLN2) was the functional target of miR-542 in glioma. In human cancers, TLN2 is known for its oncogenic function. For instance, a study has reported that TLN2 was up-regulated in breast cancer, and that its down-regulation inhibited the growth and invasion of breast cancer cells *in vitro,* and suppressed breast cancer tumorigenesis *in vivo* [18]. All in all, miR-542 regulated the growth of glioma cells by targeting TLN2. This finding underscores the therapeutic importance of the miR-542/TLN2 molecular axis.

CONCLUSION

Glioma cells expressed significantly low miR-542 transcript levels. The re-expression of miR542 inhibited the proliferation of glioma cells via induction of apoptosis. At the molecular level, miR-542 inhibited glioma cell growth by targeting TLN2 post-transcriptionally. Overall, the results obtained in this study are suggestive of the possible diagnostic and therapeutic importance of miR-542 in glioma.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jinlan Li and Jiapeng Mo contributed to this work equally.

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