Involvement of \textit{EGFR}, \textit{ERK-1,2} and \textit{AKT-1,2} Activity on Human Glioma Cell Growth

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Abstract

GBM (Glioblastoma multiforme) is the most prevalent and lethal primary brain tumor. Gene therapy is one of the promising approaches and involves the delivery of genetic therapeutic molecules for specific antitumour response/activity. miRNAs can regulate the cell biology functions including replication, cell growth, and apoptosis by regulating gene expression. In this study, we found that down-regulation of miR-4731 expression occurred in GBM cells. We further determined that miR-4731 behaved as a tumor suppressor by inhibiting GBM cell proliferation. We further investigated the molecular mechanisms of miR-4731 and \textit{EGFR}, \textit{ERK-1,2} and \textit{AKT-1,2} in GBM cell lines U87 and U251. The \textit{in vitro} ectopic expression of miR-4731 affected cell proliferation, migration, and invasion of U87 and U251 cells. Luciferase reporter assays validated that miR-4731 targeted the 3′-untranslated region (3′-UTR) of \textit{EGFR}. In conclusions, we identified that miR-4731 plays a tumor suppressor role in GBM cell proliferation and migration by targeting \textit{EGFR} expression, and miR–4731 may act as a novel biomarker for early diagnosis or therapeutic target of GBM.

Keywords: Glioblastoma- cell cycle- miR-4731- miRNAs

Introduction

GBM (Glioblastoma multiforme) is the most prevalent and lethal primary brain tumor (Ohgaki and Kleihues, 2013). The standard treatment method involves maximal resection of the tumor followed by radiation and chemotherapy (Wesseling and Capper, 2018). Gene therapy is one of the promising approaches and involves the delivery of genetic therapeutic molecules for specific antitumour response/activity (Manikandan et al., 2019). MicroRNA (miRNA) is a kind of non-coding RNA with the length of 19-23 nucleotides, which binds to the 3′ UTR of mRNA of the target genes to regulate the expression per se/their gene expression. miRNAs can interfere in transcription process of RNA and inhibit the expression of target genes (Valinezhad Orang et al., 2014). Many miRNAs have been known to perform specific functions in the regulation of tumor progression and multiple drug resistance (Shirjang et al., 2019). The 256 miRNAs were found to be increased and 95 miRNAs were reported as downregulated in GBM (Johnson et al., 2005).

miR-4731 has been demonstrated to be a novel miRNA, which has been characterized as a tumor suppressor in human melanoma (Stark et al., 2016). However, the specific roles and regulatory mechanisms of miR-4731 in the progression of GBM have not been explored yet. Additionally, \textit{EGFR} expression was identified to be highly expressed in a variety of human cancers, such as GBM (Huang et al., 2015). However, it is not clear whether miR-4731 can regulate the expression of \textit{EGFR} in GBM.

In this study, we investigated the regulation of \textit{EGFR}, \textit{ERK-1,2} and \textit{AKT} expression by miR-4731 and their effects on proliferation, cell cycle transition and invasion in glioblastoma cell lines U-251 and U-87. Our results may provide data for supporting miR-4731 as novel therapeutic tools for glioblastoma multiforme.

Materials and Methods

Bioinformatics analysis

The KEGG database (https://www.genome.jp, RRID:SCR-012773) was obtained to accurately identify...
genes involved in the EGFR pathway. The target genes of miR-4731 were selected based on target scan algorithms. Expression Atlas database (https://www.ebi.ac.uk/gxa/home) was studied to determine the expression levels of EGFR, ERK and AKT genes in U-251MG and U-87MG cell lines.

Cell culture and virus production

Glioma cell line U-251MG was gifted from Stem Cell Technology Research Center (Tehran, Iran) and glioma cell line U-87MG (IBRC C10982) was purchased from Iranian Biological Resource Center (IBRC) (Tehran, Iran). Human embryonic kidney 293 T (IBRC C109683) was purchased from the Iranian Biological Resource Center (IBRC) (Tehran, Iran). All cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM, Bio Idea, Tehran, Iran) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and 10% fetal bovine serum (FBS; Gibco). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. For Virus production, HEK293 T cells were seeded in T-25 flask at 60–70% confluency. Virus packaging was performed using PEI transfection reagent (Polysciences, USA) according to the manufacturer’s protocol. hsa-miR-4731 recombinant vector and scramble vector co-transfected separately with psPAX and pMD2 vectors (psPAX and pMD2 virus packaging helper vectors were purchased from Stem Cell Technology Research Center (Tehran, Iran)) to HEK293 T cells and the culture media containing recombinant viral vectors was collected for 48, 72 and 96 hours. For in vitro functional assays, glioblastoma cells were seeded in T25 flask at 60–80% confluency. Transduction was performed by using Hexadimethrine bromide (commercial brand name Polybrene) as a transduction enhancer.

Vectors and microRNA

The miR-4731 gene was amplified by PCR using specific primers (forward: 5'-GCTCTAGACAAAGATTCTACCCAAGCACAAG-3'); reverse: 5'-CGGGATCCATAGCGACGCTATAGAGTTG-3'). This fragment was cloned into the pCDH-CMV-MCS-EF1-cGFP-T2A-puro vector (System Biosciences, USA). The 3'UTR sequence of EGFR gene was amplified by PCR from genomic DNA using specific primers (forward: 5'-CCGGTCTGAGCCAAGAGATAATGTCG-3'; and reverse: 5'-ATAGGAATGACGGCCGCCTTCTTAAAATCAGCCTAGG-3'). These fragments were cloned into the psiCHECK-2 dual-luciferase reporter plasmid (Promega, Madison, WI, USA) downstream of the renilla luciferase gene.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

After 72 hours, total RNA from transduced glioma cells (U251 and U87MG) was extracted using GeneAll (Hybrid-R™) kit. The quality and concentration of total RNA were evaluated by spectrophotometry. To quantify EGFR, ERK-1/2 and miR-4731 expression, M-MLV reverse transcriptase (Thermofisher) was used for synthesis of single-stranded cDNA, and RT-qPCR was conducted using SYBR Green. miR-4731 expression was calculated relative to SNORD47 gene, and EGFR, ERK-1/2 and AKT expressions were calculated relative to β2M gene. The primers in this study used for cDNA synthesis and real-time PCR were as follows in Table 2. Relative expression was evaluated by the 2-ΔΔCt method.

Luciferase reporter assay

For the luciferase reporter technique, miR-4731 or scramble vector was transfected into hek-293T cells, along with EGFR-3'-UTR using PEI transfection reagent (Polysciences, USA) according to the manufacturer’s protocol. Luciferase activity was evaluated at 48 h post-transfection by a Dual-Luciferase Reporter assay system (Promega), considering to the manufacturer’s instructions. Firefly luciferase activity was obtained for normalization.

Flow cytometry analysis

Cell cycle was analyzed 72 hours after transduction. Transduced cells were collected by trypsinization, washed in cold PBS and fixed with paraformaldehyde. Then permeabilization of cells was performed with cold 70% ethanol, stained cells with dye including PI, PBS, Triton X100 and RNase A. After incubation at 37°C in the dark for 30 min, we assessed the samples on the flow cytometer BD FACS Calibur (BD Biosciences, San Jose, CA, USA).

Colony forming assay

Briefly, U-87MG and U-251MG cells were seeded in 6 cm dishes at a concentration of 1,000 cells/well until colony formation. After 15 days, the supernatants were removed; then, colonies were carefully washed with PBS, fixed with cold methanol for 20 min, and stained with crystal violet 0.1% in PBS at room temperature for 10 min and air-dried. Images were captured and the total number of colonies/well was counted.

Western blot analysis

Cells transduced with LV-miR-4731 were collected, washed twice with cold phosphate-buffered saline (PBS) and resuspended in the RIPA lysis buffer with protease inhibitors (Roche Diagnostics GmbH) phosphatase inhibitors (Merck KGaA, Darmstadt, Germany). The total proteins was quantified using a BCA Protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The same values of each protein sample were electrophoersed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (PVDF) (EMD Millipore, Billerica, MA, USA) and blocked with 5% fat-free milk. The membranes were then incubated overnight at 4°C with primary antibodies: Rabbit polyclonal Anti-ERK1,2 (EPR17526), Rabbit Anti-AKT1 + AKT2 + AKT3 antibody (ab126811) and Rabbit anti-human monoclonal Actin antibody (ab8227; 1:1000 dilution; Abcam; Cambridge; MA; UK). After washing three times with Tris-buffered saline with 0.5% Tween-20 (TBST; Beyotime Institute of Biotechnology, Haimen, China), the membranes were probed with Goat Anti-Rabbit IgG H&L (HRP) (ab6721; 1:5,000 dilution;
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Abcam; Cambridge; MA; UK) at room temperature for 1 h. The protein blots were visualized using the ECL Protein Detection kit (Pierce Biotechnology). The signal was detected using an enhanced chemiluminescence western blotting detection system.

**Statistical analysis**

The data in this study are presented as mean ± SD. Data were compared using Student’s t-test with GraphPad Prism 5.0 software and real-time PCR data was analyzed statistically by $2^{-\Delta\Delta C_{t}}$ method. Differences were assessed significant at $P<0.05$.

**Results**

**miRNA and gene selection**

EGFR, ERK-1,2 and AKT, were selected as target genes according to the KEGG database. hsa-miR-4731 that is targeted these genes was selected based on TargetScan algorithms. Moreover, based on Expression Atlas database, expression of EGFR, ERK-1,2 and AKT-1,2 in U-251 and U-87 cell lines were medium. In order to compare the expression level of the genes in the expression Atlas database, cerebral cortex was selected as non-tumoral tissue. The results of these evaluation indicated that the expression level of these genes naturally in tumoral cells (U251MG and U87MG) is higher than non-tumoral cells (Figure 1).

**Induction of miR-4731 expression in glioblastoma cells**

After transfection of HEK293 T, LV-miR-4731 was collected and used for transduction of U-87MG (Figure 2A) and U-251MG (Figure 2B) cell lines of glioblastoma. Then the expression of miR-4731, EGFR, ERK-1,2 and AKT-1,2 in two cell lines of glioblastoma (U-87MG and U-251MG) were evaluated by real-time PCR and compared with the glioblastoma cells transduced with the scramble vector as the control. The results showed the expression of miR-4731 in two cell lines and not in scramble group (Figure 2C, D).

**Expression of EGFR, ERK-1,2 and AKT-1,2 in glioblastoma cell lines**

we assessed expression of EGFR, ERK-1,2 and AKT-1,2 in U-251MG and U-87MG cells after 72 hours transduction with Lv-miR4731. In the independent experiments we observed marked reductions of EGFR, ERK-1,2 and AKT-1,2 transcript in glioma cells in response to miR-4731 infection (Figure 3). Of note, the EGFR mRNA levels were not decreased by miR-4731 over expression in U-87MG.

**miR-4731 directly targets and inhibits EGFR**

After the downregulation of EGFR expression as a result of the overexpression of miR-4731 based on real-time PCR and the prediction of both of the genes targeting by miR-4731, luciferase reporter assay was performed to validate the binding miR-4731 and 3’-UTR of the EGFR gene. These findings indicated that overexpression of miR-4731 can reduce the expression of the EGFR to ~66% ($p < 0.0001$). Therefore, it is

**Table 1. Primers Used for cDNA Synthesis and Real-Time PCR for EGFR, AKT,1,2 and ERK-1,2 Genes and hsa-miR-4731-5P.**

| Gene  | Forward | Reverse |
|-------|---------|---------|
| EGFR  | CGTCCGCAAGTGTAAAGAAG | AGGAGTCACCCTAATGC |
| AKT1  | TGGCACTTCTATTGGCTAC | GTCTGATGCGGTTGTC |
| AKT2  | ATGGCAAGGATGAACTCG | CTACAAGAGCCGAGCACAATC |
| ERK1  | GGTTGAGATGTGAAGGG | GCAAGCAGATCTGGATC |
| B2M   | ATGCCCTGGTGTAAC | ATCTGAAACCTCAGTAC |
| Snord 47 | GAGCAGGGTCCAGGT | GAGCAGGGTCCAGGT |
| Common Reverse primer | GAGCAGGCTCAGGTCAGGT | GAGCAGGCTCAGGTCAGGT |
| hsa- miR-4731-5p | GTCGATCGAACAGGAGGTTTCGGAGGATTCGCACTGCATAAGACACTC | GTCGATCGAACAGGAGGTTTCGGAGGATTCGCACTGCATAAGACACTC |
suggested that miR-4731 directly targets and inhibits EGFR expression. (Figure 4).

miR-4731 regulates proliferation of glioblastoma cells

To investigate the effects of miR-4731 on the proliferation of glioblastoma cells, by using colony formation assays, we observed that transduced U-251MG and U-87MG cells with miR-4731 dramatically decreased the growth rate of both types of glioblastoma cells as compared with that of the non-transduced cells. miR-4731 decreased the growth rate of both types of glioblastoma cells as compared with that of the non-transduced cells (Figure 4A and B). This suggests that up-regulation of miR-4731, which results in EGFR, ERK-1,2 and AKT-1,2 suppression, decrease the proliferation of glioblastoma cells (Figure 5).

Overexpression of miR-4731 induces cell cycle arrest in glioblastoma cells

To investigate the effect of miR-4731 on the...
proliferation of glioblastoma cells, we transduced the glioblastoma cells with \( LV-miR-4731 \) to increase its expression in these cells, then we analyzed the cell cycle by flow cytometry method. The miR-4731 resulted in significant increase in number of the cells in G0/G1 phase and reduces of cells in S phase (Figure 6). Our data indicate that miR-4731 induce G0/G1 cell cycle arrest in GBM cells.

**Downregulation of ERK-1,2 and AKT protein in glioblastoma**

The results of Western blot showed that the ERK-1,2 and AKT protein were down-regulated in U-87 MG and U-251MG cells transduced with LV-miR-4731 in comparison of miR-control groups. Our findings revealed that, ERK-1,2 and AKT protein expression decreased in the U-87 MG and U-251MG cells transduced with LV-miR-4731 compared with the control group (Figure 7).

**Discussion**

Despite significant advances made over last years on molecular mechanisms invasion in glioblastoma, the exact reasons are still unknown and need more research. The treatment options for patients diagnosed with GBM are limited and the current median survival is 12-14 months following diagnosis (Kanu et al., 2009). Previous studies have shown that GBM frequently deliver mutations that activate EGFR and launch downstream signaling...
pathways, including the AKT and ERK molecules (Mazzoleni et al., 2010). The EGFR signaling is activated in 60% of glioblastoma tumors and so, being a ‘critical molecule’ for glioblastoma (Maire and Ligon, 2014). The extracellular signal-regulated kinases (ERKs) is a signal transducer growth factor to the nucleus and involved in a wide range of biological responses, including cell proliferation, differentiation and motility. ERK1/2 is aberrantly expressed and activated in glioblastoma multiforme (Sun and Nan, 2017). AKT is a serine/threonine kinase in and large scale genomic analysis of GBM, has been demonstrated that this molecule is mutated in the majority of GBMs and inhibition of AKT an attractive target for GBM therapy (A McDowell et al., 2011).

The miRNAs can bind with 3'-UTR region of target gene and suppress the transcription of target gene. In cancers, the miRNAs expression patterns have been changed and alter the proliferative signaling, cell growth, invasion, and angiogenesis. EGFR pathway can also be regulated by many miRNAs. For example, miR-7 is down-regulated in human glioblastoma and directly targets EGFR expression. In addition, miR-7 suppresses Akt and ERK pathway activation independent of its EGFR inhibition (Liu et al., 2014).

The present study indicated that the expression level of the EGFR, ERK-1,2 and AKT-1,2 in glioma cell lines is medium and can induce cell growth in these cells. Additionally, increased expression of miR-4731 significantly correlated with reduced expression of EGFR, ERK-1,2 and AKT-1,2 at the mRNA levels and ERK-1,2 and AKT-1,2 at the protein levels. The difference in expression-eduction of EGFR, according to RT-qPCR in U-87 cell line is probably due to EGFR not mutated in this cell line (Patil et al., 2015). According to these results, We found that overexpression of miR-4731 induced to increase the sub-G1 population in glioblastoma and leads to G1 arrest in cell cycle.

miR-4731 was previously reported to be suppressed in melanoma and following overexpression of miR-4731, multiple genes associated with cell cycle were regulates (Stark et al., 2016). In the present study, Through bioinformatics analysis, EGFR was predicted to contain a miR-4731 seed match at position 541-548 of the EGFR-3'UTR. To test whether miR-4731 directly targets 3'UTR of EGFR, we performed luciferase reporter assay. By measuring changes in the luciferase activity, we proved that miR-4731 directly targets 3'UTR of EGFR. In this study, we found new target for miR-4731 that may affect cell cycle. EGFR and downstream molecules such as AKT and ERK activate many biological outputs that are beneficial to cancer cell proliferation and progression through the cell cycle (Wee and Wang, 2017). Our study demonstrated that miR-4731 overexpression could decrease the colony formation in glioma cells compared with the control groups. The present data indicated that miR-4731 attenuated cell proliferation in glioma cell lines.

In conclusion, the findings of the present study identified miR-4731 reduces EGFR, AKT-1,2 and ERK-1,2 expression in glioblastoma cell lines and induce cell cycle arrest and inhibit cell growth in these cells. Thus, miR-4731 overexpression may be a potential therapeutic strategy in combination with conventional therapy for the treatment of glioblastoma.

Acknowledgments

The authors would like to thank the National Institute for Medical Research Development (NIMAD: Grant No. 942974) and Tehran University of Medical Sciences (Grant No. 94-04-87-36877) for the financial support of this work.

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