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The *MiR-561* Suppresses Glioblastoma Cell Proliferation through C-myc Regulation

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Abstract

Background: Glioblastoma multiforme (GBM) is the most common primary malignat brain tumor in adults. The modulation of *miRNA* expression is considered both as controlling groundwork for cancer development and invasion and as a potential application in GBM-targeted therapies either perse or combined with chemo-or radiotherapy. The c-myc overexpression is tightly correlated with GBM progressing growth and malignancy. There is ample evidence showing that microRNAs (miRNAs) are linked to the pathogenesis of several malignancies. However, little is known about the potential role of miRNAs in GBM development. We conducted the present study to find out whether the *miR-561* inhibits GBM cells proliferation and survival via controlling the expression of c-myc.

Method: In this in vitro study, the U87 cell line was used as a template for lentiviral vector "*pCDH-miR-561*" construction. HEK293 cell line was transfected with *pCDH-miR-561* and its viability (MTT assay) and apoptosis rates (flow cytometry) were monitored. c-myc expression was monitored employing q-RT PCR. In order to search for possible *miR-561p* targets, we utilized bioinformatics tools of TargetScan and DAVID.

Results: Our results confirmed that the overexpression of the *miR-561* inhibits cell proliferation and promotes cell apoptosis in GBM cancer cells, which is tightly correlated with the downregulation of c-myc.

Conclusion: These findings proposed that the *miR-561* has promising qualifications to suppress U87 growth and proliferation via tuning the c-myc, which then makes it a useful model for GBM treatment.

Keywords: Neoplasms, Glioblastoma, MicroRNAs, *miR-561*, c-myc

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Introduction

c-myc oncogene encodes a conserved basic helix-loop-helix (HLH) leucine zipper transcription factor that regulates a broad range of cellular processes, including cell cycle, apoptosis, cellular differentiation, cellular metabolism, and genomic instability.[1-4] c-myc regulates the expression of 10%-15% of the genes encoding, both proteins and non-coding RNAs in the genome, which mediates its biological functions.[5] In a normal situation, the expression of c-myc gene is tightly controlled at certain phases of the cell cycle. Therefore, any changes in the expression of c-myc would induce further genomic instability, unresponsiveness toward growth factors (immortalization), and development of various strategies to escape immune surveillance, which eventually results in tumor formation.[6-8] Even though the overexpression of c-myc in tumors as a matter of chromosomal translocation, gene amplification or stabilization of its mRNA has been well studied, the role of micro RNAs (*miRNAs*) as mediators that are involved with c-myc expression profile has not been well stablished.

The *miRNA*s are small non-coding RNAs. They regulate post transcription of several genes, including those involved in the cell growth, differentiation, proliferation, apoptosis, and cell death.[9-17] The deregulation of various *miRNA*s normally correlates to various cancers.[18-21] It then points out the important role of *miRNAs* in tumor progression. The *miR-561*, as a tumor suppressor, downregulates in several cancers, for instance gastric cancer, colon cancer, and glioblastoma. The in vitro studies on gastric cancer tumor invasion revealed that the *miR-561* inhibits cell proliferation via regulation of c-myc expression.[22-23]

Glioblastoma multiforme (GBM) is the most lethal form of brain cancer with a poor prognosis and low survival rate in patients. Recently, there is further focus on the investigation of molecular mechanism of GBM to improve the efficiency of applied treatments.[24,25] Hence, the modulation of *miRNA* expression is considered both as controlling groundwork for cancer development and invasion and as a potential application in GBM-targeted therapies either perse or combined with chemo-or radiotherapy.[24-29] There are several reports claiming that c-myc overexpression tightly correlates with GBM tumors. If c-myc overexpresses individually, the tumor grows very slowly compared to the time when c-myc overexpresses simultaneously with other oncogenes (e.g. *Ras* and *Akt*) and tumor suppressor genes (e.g. *p53* and *Ink4c*).[30,31] Furthermore, the overexpression of c-myc have been found to initiate different types of brain tumors, like medulloblastoma,[32,33] pancreatic neuroendocrine tumors (PNETs),[34,35] and glioma[34-39] in mice.

The overexpression of the c-myc in tumors and its correlation with chromosomal translocation, gene amplification, and its mRNA stabilization have been well studied. Considering the recognized functions of c-myc in both normal and malignant cells, the ability of the *miR-561* to inhibit GBM cells proliferation and survival via controlling c-myc expression was investigated. We found that the overexpression of the *miR-561* inhibits U87 cell proliferation and promotes cell apoptosis in GBM cancer cells. Further analysis confirmed that the *miR-561* targets indirectly controlled c-myc expression.

Material and Methods

In the current in vitro study, the *miR-561* encompassing sequence was primarily retrieved from U87 cell genomic DNA. Secondly, HEK293 cell line was transfected with *pCDH-miR-561* and its viability and apoptosis rates were monitored while c-myc expression was monitored simultaneously. In the end, to find possible *miR-561p* targets, bioinformatics tools were used.

Cell culture condition

The U87 (GBM cell line) and HEK293 (Human Embryonic Kidney 293 cell line) cells were maintained in Dulbecco's Modified Eagle's medium (DMEM). All the media were supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and 1% antibiotics. The cells grown in in a humidified incubator with a 95% air/5% CO2 atmosphere at 37°C.

Plasmid and viral vector construction

The design of the *miR-561* plasmid construct is described in this section. The *miR-561* encompassing sequence was amplified with polymerase chain reaction (PCR) using U87 cell genomic DNA as template and cloned between the cutting sites of *EcoRI* and *BamHI* restriction enzymes in the *pCDH-TurboGFP* plasmid. Empty vector of *pCDH (pCDH-Ctrl)* was utilized as the negative control in all the experiments. For lentiviruses packaging, *pCDH-miR-561/pCDH-Ctrl, psPAX,* and *pMD2* co-transfected to HEK 293 cell line using calcium-phosphate methods. The supernatants of the cells were collected every 12 hours and concentrated by ultracentrifuge at 20.000 rpm for two hours.

RNA extraction and qRT–PCR

Total RNA was extracted from all the groups of U87 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Two microgram of total RNA was used as the template. Subsequently, both M-MLV Reverse Transcriptase (Promega, USA) and random hexamers were used after setting mRNA as the template. We employed the BONmiR kit (Stem Cell Technology Research Center) for the experiments in which *miRNAs* was the template. The procedures were performed according to the manufacture's instruction. We carried out Real time PCR with the reagents of a SYBR green I mix (Takara, Japan) in a 20 ml reaction volume. The data were normalized with HPRT gene (for *mRNAs*) and SNORD47 (for *miRNAs*) and analyzed using the $2^{-\Delta\Delta Ct}$ method.

Cell viability and apoptosis assays

Cell viability was assessed utilizing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The U87 cells were seeded in 96-well plate and transduced with the *miR-561* containing lentiviruses. Following the transduction, the MTT dye with concentration of 5 mg/ml was added to each well of a 96-well plate. The cells were then cultured for four hours by the interval times of 48, 72, and 96 hours. The culture medium, which was mixed with MTT dye, was discarded and 100 ml of dimethyl sulfoxide (DMSO) was added to each well. The

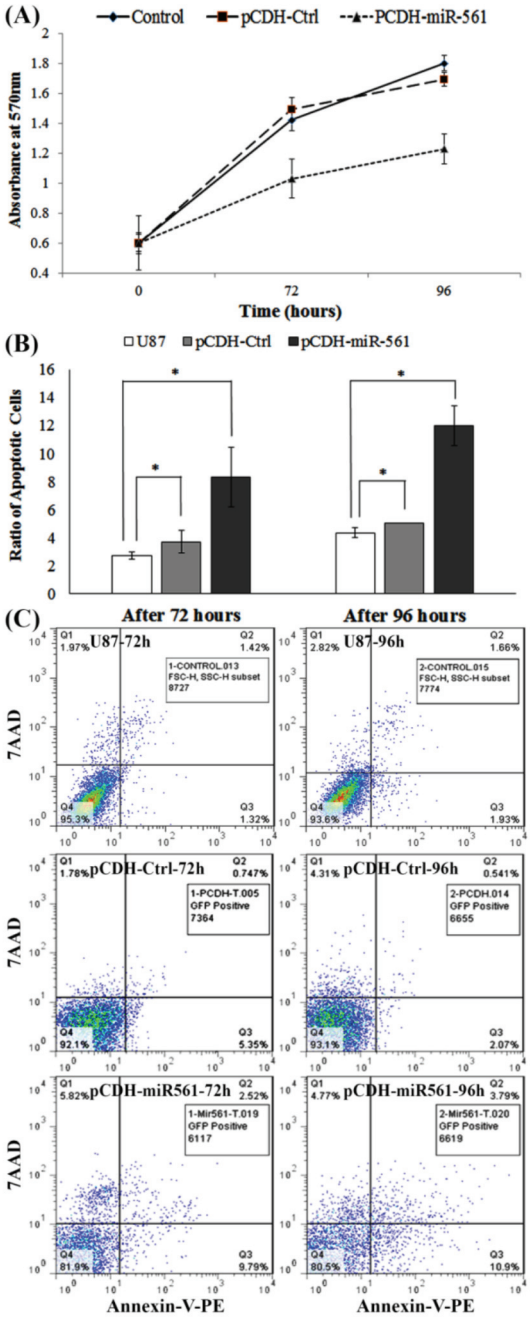

Figure 1. The *miR-561* induces apoptosis in GBM cells. (a) *pCDH-miR-561* was transduced to U87 cells in 96-well plate. The cell viability was assessed at 72 and 96 hours after the transduction using MTT assay. (b) The apoptotic rate of the U87 cells was measured with flow cytometry. (c) Apoptotic rate in the *miR-561* transduced cells compared to the controls. The 7-AAD at Y-axis stands for "7-amino-actinomycin D" fluorochrome, while X-axis represents Annexin V – FITC intensity. The data indicate the averages of the three independent runs and are represented as bars. The error bars show the standard deviations. The "*" star demonstrates significant differences with *P*-values less than 0.05.

absorbance of each well was measured at 550 nm using a multi-well spectrophotometer (Bio-Tek, USA).

Cell apoptosis was also measured with an Annexin V-PE apoptosis detection kit (BD, USA) according to the manufacturer's protocol and its correspondingly was assigned applying flow cytometry method (BD, USA).

Cell-cycle analysis

A population of 105 cells was seeded into 6-well plate and transduced with control Lentiviral vector *(pCDH-Ctrl)* and the *miR-561* virus particles *(pCDH-miR-561)*. The viable cells were harvested and counted at 48, 72, and 89 hours

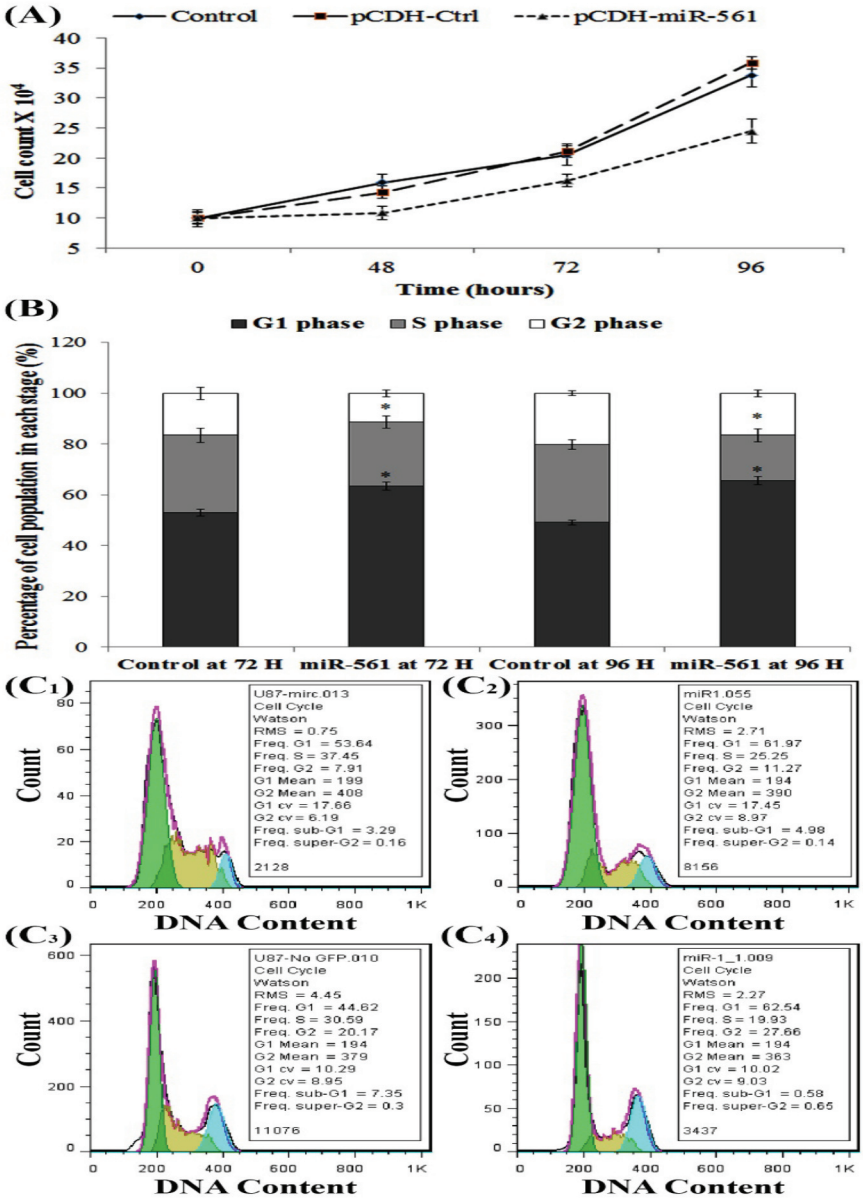

Figure 2. Ectopic expression of the *miR-561* induces G1 arrest. (a) Cell growth curve drawn for U87 transduced cells within 96 hours. The points are the average of three separate experiments. (b) Transduced U87 cells were collected after 72-96 hours and cell cycle distribution was examined using flow cytometry; (c) Frequency diagram of U87 cells accumulation in different cell phases. The c1 to c4 stand for Control at 72h, the *miR-561* at 72h, Control at 96h and the *miR-561* at 96h, respectively. The closer the amount of RMS is to zero, the closer the estimation of convoluted (pink) graph would be to deconvoluted (blue, dark, and pale green) graphs. The rest of the information is calculation details. The data were the averages of at least three independent runs; error bars represent standard deviation. The "*" star demonstrates significant differences with *P*-values less than 0.05.

following the transduction using ViCell counter and trypan blue exclusion method (Beckman Coulter).

For cell cycle analysis, *pCDH-miR-561* or *pCDH-Ctrl* lentiviruses were transduced to U87 cells, harvested at different time points (for 3 days, each 24 hours), and evaluated using flow cytometry. The distribution of cell-cycle phases was performed by propidium iodide staining and flow cytometry (BD Biosciences, San Jose, CA, USA).

Statistical analysis

The results were obtained through averaging three independent experiments and presented as the mean ± standard deviation (S.D.). We performed the statistical analysis using one-way ANOVA. *P*-values less than 0.05 were considered to be significant.

Bioinformatic analysis

In order to search for possible *miR-561*p targets, we employed the online tool for prediction of differentially expressed genes (http://www.targetscan.org/vert_71/).[36] The standard cut-off for screening predicted miRNAs was 8 mer, at the most; meanwhile, the percentage of context ++ score (CS) should not be less than 95%. The CS is the cumulative sum of 14 features. The list of the *miR-561-p* targets, including 3407 items, was downloaded and converted to Entrez-gne-ID. Afterwards, they were submitted to database for annotation, visualization, and integrated discovery (DAVID) v6.8.40. In the ultimate step, the annotation summary results of the *miR-561-p* target list were retrieved.

Results

To examine the biological impact of the *miR-561* in GBM, the *miR-561* was overexpressed following *pCDH-miR-561* construct transduction in U87 cell line. The *pCDH* plasmid was also transduced into U87 cells separately, as control construct, and named as *pCDH-Ctrl*. Cell viability, apoptosis, and cell cycle analysis were examined after the transduction of either constructs.

Impact of the miR-561 on viability of U87 cell line

As shown in figure 1a, the overexpression of the *miR-561* resulted in a significant diminution of absorbance in MTT assay, in comparison with both of non-transduced cells (Control) and p-CDH transduced cells *(pCDH-Ctrl)*. It then expressed diminished viability of U87 cells which were transduced with *pCDH-miR-561*, compared with *pCDH* transduced cells *(pCDH-Ctrl)*.

The miR-561 induces apoptosis in U87 cell line

The apoptotic impact of the *miR-561* on U87

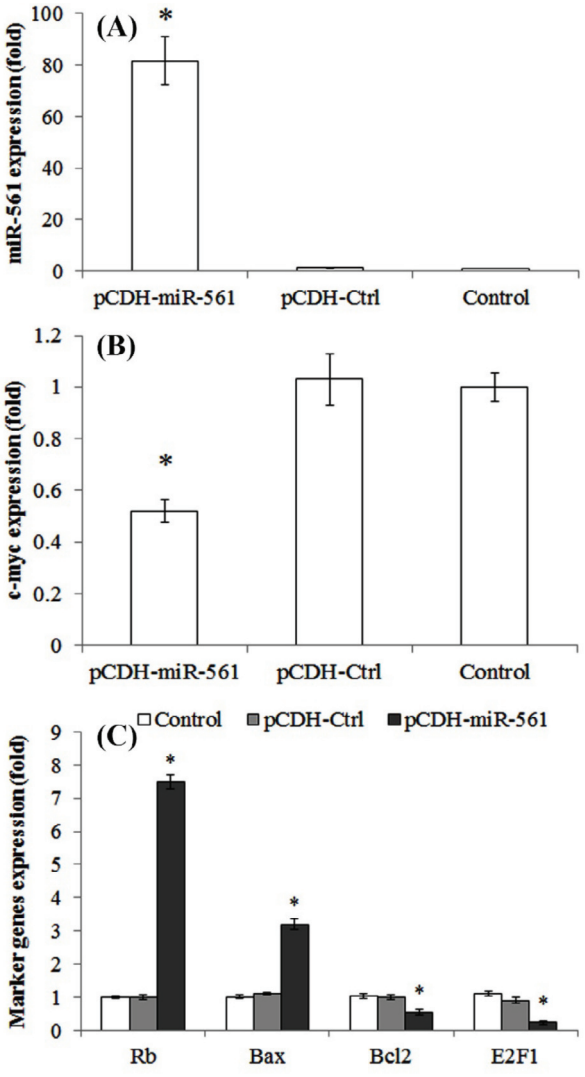

Figure 3. The overexpression of the *miR-561* modulates mRNA level of the involved genes in both apoptosis and cell cycle pathways. (a) The expression of the *miR-561* in the U87 cells was measured with QRT-PCR following transduction with *pCDH-Ctrl* and *pCDH-miR-561*. The results of *SNORD47* gene as an endogenous control; (b) c-myc is downregulated in *miR-561* transduced U87 cells. The results of *HPRT* gene as an internal control; (c) The overexpression of *miR-561* downregulates *BCL*[2] and E2F1 whereas it upregulates *BAX* and R*b* genes. Each bar represents the average of three different experiments.

cells was examined using Annexin-V-PE apoptosis detection kit. The overexpressed *miR-561* raised the apoptotic rate of U87 cells by 8.3- and 12-fold at 72 and 96 hours of post-transduction, respectively (Figure 1b-c). However, we observed no elevated apoptotic rates up to 48 hours in any of the cases. According to Q4 section of figure 1C, which represents the population of normal cells, 94.9% of U87 cells, 92.7% of *pDCH-Ctrl* cells, and 82.7% of *pCDH-miR561* were normal after 72 hours. The aforementioned percentages dropped to 93.4%, 90.2%, and 83.4% by 96 hours, respectively. According to Q3 section of figure 1C, the population of early apoptotic cells increased by 7.07 and 3.22 folds, respectively, after 72 and 96 hours of transfection with *pCDH-miR561*. Finally, considering Q2 section, after 72 and 96 hours following the transfection of U87 cells with *pCDH-miR561*, the population of late apoptotic, necrotic, and dead cells increased by 2.62 and 3.04 folds, respectively. Therefore, the highest prevalence of early apoptotic and dead population of *miR561*-transfected cells were observed after 72 and 96 hours, respectively.

Impact of the miR-561 on cell cycle regulation

In order to investigate the impact of the *miR-561* on cell growth, the cell proliferation curve of *pCDH-miR-561* transduced U87 cells was examined. As could be seen in figure 2a, the U87cell proliferation rate markedly reduced in the *miR-561* overexpressed group compared with the control groups. Subsequently, the effects of the *miR-561* upregulation on cell cycle distribution were analyzed in the U87 transduced cells using flow cytometry. The results indicated that the *miR-561* triggered further accumulation of U87 cells at the G1 stage (10.4% increase in 48 hours and 16.4% increase in 96 hours)t; whereas, the population of cells in S-phase stage declined by 5.1% and 12.6% in 48 and 96 hours, respectively (Figure 2b, c). We did not observe any significant changes in the population of the cells in G2/M stage neither in 48 hours nor in 96 hours (Figure 2b, c).

How the miR-561 has an impact on qRT-PCR markers

Since the *miR-561* has important targets in cell cycle regulation, we were wondering whether it could change downstream biological effects of cell cycle, including c-myc gene expression.

To analyze whether the *miR-561* regulates c-myc expression, U87 cells were transduced by *pCDH-miR-561* lentiviruses. The results revealed that the overexpression of *miR-561* (by 80 folds) resulted in a significant decrease (more than 50% decline) in endogenous c-myc transcript (Figure 3a, b).

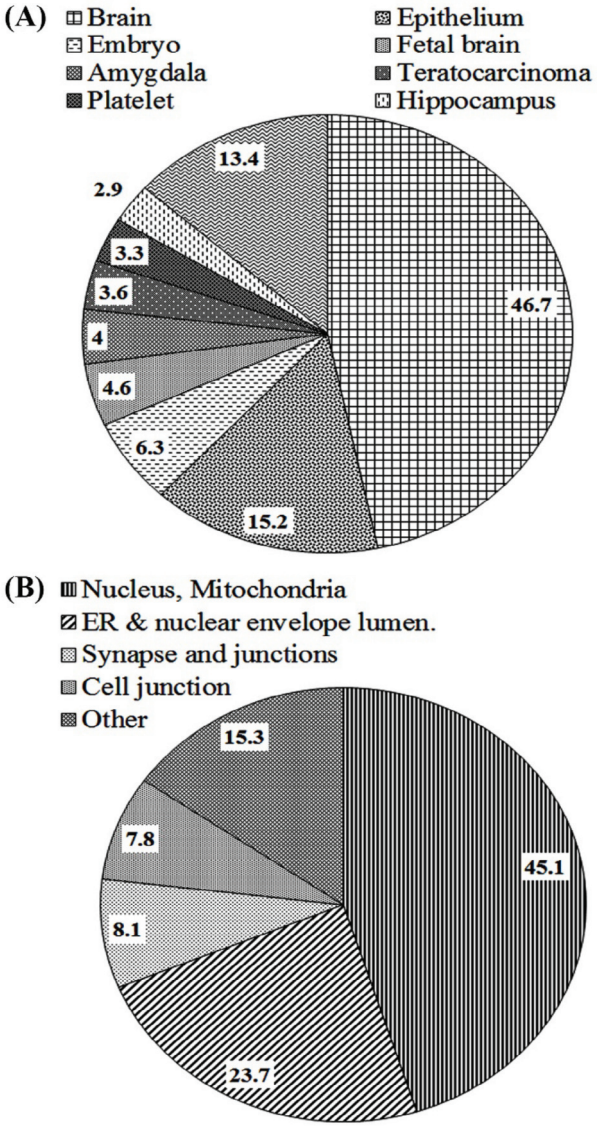

Figure 4. The *miR-561* targets. (a) Tissues that the *miR-561* targets the most. The numbers in the pie graph show the corresponding percentage of each tissue; (b) Subcellular organelles that the *miR-561* targets the most.

The quantitative RT-PCR results confirmed that the overexpression of *miR-561* induced the upregulation of proapoptotic *Bax* gene and the downregulation of anti-apoptotic BCL_2 gene by 3.2 and 0.55 fold, respectively (Figure 3c). The *miR-561* overexpression also changed cell cycle regulatory genes, such as Rb1 (by 7.49 Fold increase) and E2F1 (by 4 Fold decrease) (Figure 3c). Therefore, our qRT-PCR confirmed that the *miR-561* was not only involved in apoptosis, but also suppressed U87 cell growth via the inhibition of proliferation.

How the miR-561 targets interact

The *miR-561*p targets, which were found and

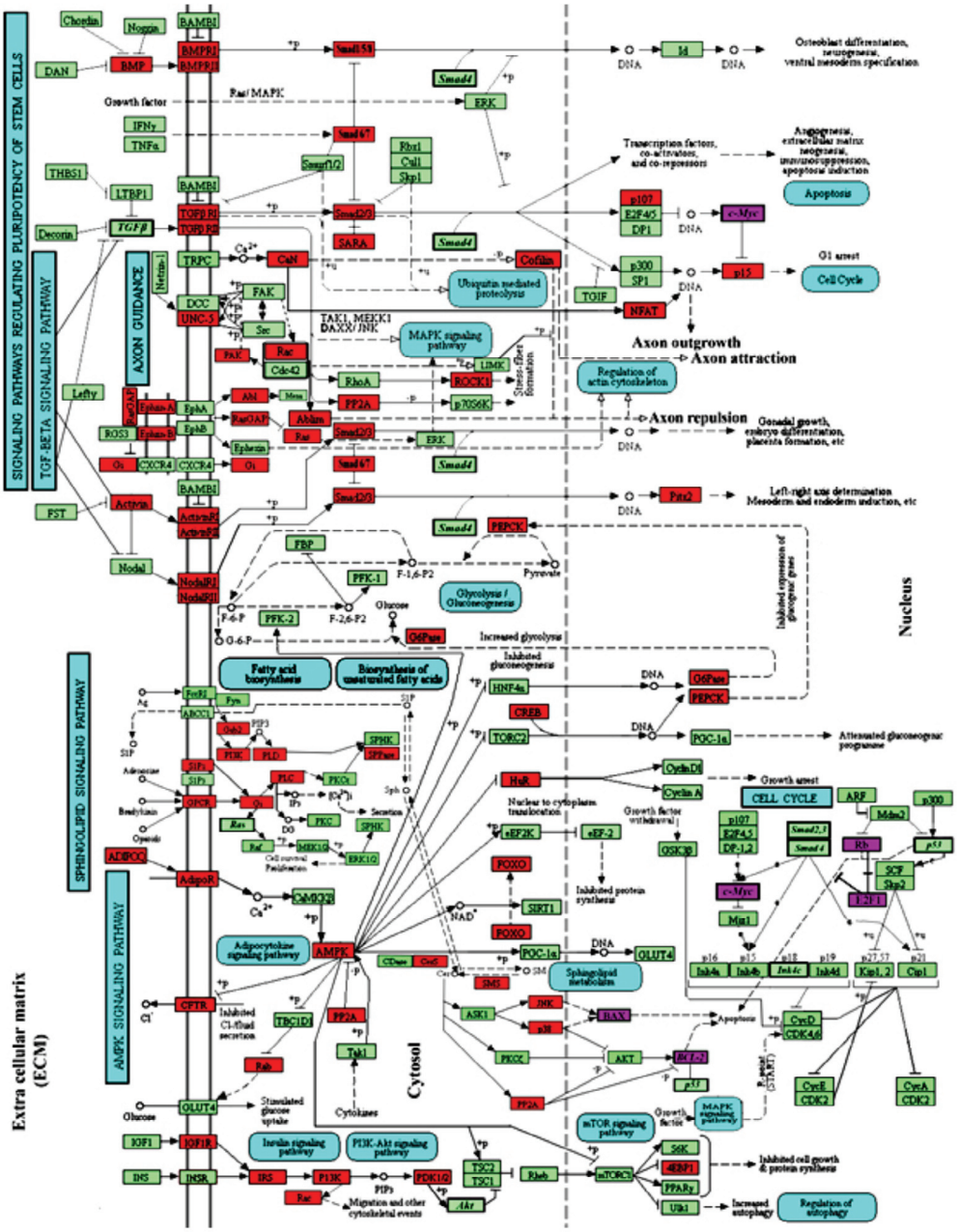

Figure 5. Cross-Referencing of pathways involved in GBM. The red marked proteins are the *miR-561* target-scan hits. The bold italic proteins are reported to be involved in GBM (44, 45). The proteins in the violet rectangular are experimentally checked apoptosis and cell cycle markers. The pale blue rectangular represents the involved pathways, adopted from Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

categorized respectively with TargetScan and DAVID, were selected according to high-ranked gene onthology (Goterm-CC-All) by 92.9% matching score, pathways (KEGG pathway) by 32.0% matching score, and tissue expression (UP_Tissue) by 94.7% matching score.

According to DAVID retrieval, there are several genes involved in controlling the cell cycle, which have been found to be mis-regulated during GBM cancer. The majority of the tissues that are the *miR-561* targets are brain, epithelium, embryo, and fetal embryo by 46.7%, 15.2%, 6.3%, and 4.6%, respectively. It confirms that most of the *miR-561* targets are either in embryonic or adult brain (Figure 4a). The targets of the *miR-561* in brain cells are mostly found in nucleus, mitochondria, and endoplasmic reticulum by 45.1%, 23.7%, and 8.1%, respectively. This leads into the idea that most of the *miR-561* targets are involved in gene transcription or brain cell metabolism (Figure 4b).

According to TargetScan retrieval list for the *miR-561*, there is a limited number of proteins' mRNA involved in the cross-referencing KEGG pathways. The top ten proteins' mRNA that are filtered according to the CS and total sites include *PDK3* (pyruvate dehydrogenase kinase, isozyme 3), *PEPCK* (Phosphoenolpyruvate carboxykinase), *CREB* (cAMP response element binding protein) and *CREB2* from hsa04152 and hsa00010 signaling pathways, *BMPR1B* (bone morphogenetic protein receptor, type IB) from signaling pathway hsa04550, *TGFBR2* (Transforming growth factor beta-receptor type 2), *CFTR* (cystic fibrosis transmembrane conductance regulator or ATP-binding cassette sub-family C, member 7), *Rab* (Ras-related protein) and *ActivinR* from signaling pathway hsa04350, and *p15* (cyclin-dependent kinase inhibitor 2B, inhibits CDK4) from signaling pathway hsa04110. Thus, four of the proteins (for example *PDK3, PEPCK, CREB* and *CREB2*) are involved in metabolism, while six others are involved in cell growth, differentiation, and apoptosis (Figure 5).

Discussion

The obtained results herein confirmed that the overexpression of *miR-561* inhibits U87 cell proliferation and promotes cell apoptosis in GBM cancer cells. Moreover, most of the *miR-561* targets suppressed GBM cells by either controlling survival genes transcription or collapsing brain cell metabolism. c-myc was also indicated to be tuned in balance with *p15* expression, which is a target of the *miR-561* in GBM. In the following paragraphs, we discuss how the *miR-561* controls the apoptosis, rate of oncogenesis, and cell cycle arrest in GBM cancer cells.

How the miR-561 controls apoptosis

Both MTT assay and quantitative RT-PCR results confirmed that the *miR-561* was an apoptotic driving factor in U87 cells. The overexpression of *pCDH-miR-561* in U87 cells, upregulated *BAX* and *Rb* genes which in turn trigger apoptosis (Figure 3, 5). On the contrary, *pCDH-miR-561* overexpression downregulated BCL_2 and *E2F1* genes that are known as the inhibitors of apoptosis (Figure 5). Hence, *pCDH-miR-561* overexpression accelerated the apoptosis rate in U87 cells by igniting apoptotic driving proteins while suppressing apoptosis inhibitor proteins.

Furthermore, several mRNA targets, which efficiently interact with *miR-561*, control fate of brain cell toward apoptosis. These mRNA targets, including *BMPR, BMP, TGF*β*R,* and *TGF*β are the key controlling elements involved in *TGF*β, and sphingolipid signaling pathways (Figure 5). Therefore, the *miR-561* has the potential to induce the apoptosis in GBM model cells (U87) via suppression of their cell cycle control, cell differentiation, and sphingolipid metabolism.

How c-myc meddles into the rate of oncogenesis

If c-myc is individually overexpressed, the tumor grows very slowly compared with when c-myc is simultaneously overexpressed with either oncogenes (for example *Ras* and *Akt*) or tumor suppressor genes (for example *p53* and *Ink4c*).[41] The proto-oncogenic activity of c-myc could result in rapid growth of tumoric cells once the apoptotic bottleneck factors, such as *Ras, Akt,*

p53, Ink4c, and *Ink4b (p15)* are tuned on low expression. In other words, the balance between c-myc and *Ras, Akt, p53,* and *Ink4c* regulates the apoptotic or oncogenic fate of the cell (Figure 5).[42,43] The tumor proteins of *p18 (Ink4c), p53,* and c-myc inhibit cyclin D and A which are involved in G1/S and S/G2 transitions (Figure 5). Apparently, c-myc is the antagonist of *p15*. Therefore, if the *miR-561* suppresses *p15'* mRNA, there would be no further need for the overexpression of c-myc, which can result in the downregulation of c-myc and gradual cell cycle arrest. Additionally, the *miR-561* efficiently interacts with *4EBP1*, which also leads into growth arrest and suppression of cell cycle pathway and autophagy via mTOR signaling pathway.[41]

Why the miR-561 might have an impact on GBM?

The flow cytometric results suggested that the *miR-561* contributed to the induction of G1-arrest in U87 cells. On the other hand, the overexpression of c-myc have been found to initiate different types of brain tumors, like medulloblastoma,[32,33] PNETs,[34] and glioma[35,37-39] in mice. Various stages of glioma have been found to be associated with the regulation of miRNAs.[46] c-myc is the key factor of regulating cell cycle progress and cell proliferation. The other key elements in *TGF*β signaling pathway such as Smads, which interact with the *miR-561* like *p15* mRNA control c-myc function. In addition, more than half of the *miR-561* targets are in the nucleus, mitochondria or endoplasmic reticulum of the brain and central nervous system cells, which are the origin of GBM (Figure 4). Our findings implied that the *miR-561* has promising qualifications to suppress U87 via tuning the balance between *p15* and c-myc at mRNA level. These findings on U87 cell line, as a model for GBM treatment, would be useful for future studies.

Conclusion

Our findings implied that the *miR-561* has promising qualifications to suppress U87 via tuning the balance between *p15* and c-myc at mRNA level. These findings on U87 cell line, as a model for GBM treatment, would be useful for future studies.

Conflict of Interest

None declared.

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