Regulation of epidermal growth factor receptor signaling by plasmid-based MicroRNA-7 inhibits human malignant gliomas growth and metastasis in vivo

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MicroRNAs are endogenous, non-coding RNAs of approximately 20-22 nucleotides that regulate genes expression by binding to the 3' untranslated region (UTR) of targets mRNAs and play critical roles in cancer pathways. Malignant glioma is the most common and highly lethal central nervous system tumor for which little effective treatment is available over several decades. The purpose of this study was to explore the therapeutic potential of plasmid-based microRNA-7 (miR-7) for gliomas in vivo. Enhancing miR-7 levels in vitro could significantly induce cell apoptosis, and inhibit cell proliferation, cell migration and invasion. Western blotting analysis was performed, which indicated that miR-7 directly inhibited epidermal growth factor receptor (EGFR) and further antagonized the downstream protein kinases including ERK, Akt and Stat3. Furthermore, systemic administration of miR-7 encapsulated in cationic liposome resulted in glioma xenografts growth arrest and the metastatic nodules decrease effectively in a sequence-specific manner. In this study, miR-7 was applied in glioma treatment for the first time in vivo. Our findings suggested that the plasmid-mediated gene therapy with miR-7 appeared to be a promising candidate for the development of new antitumor and anti-metastasis treatment for human glioma.

Key words: miR-7, glioma, metastasis, apoptosis, gene therapy

Malignant glioma is the most common and lethal primary brain tumors in adults. The deadly nature of malignant gliomas resides in their explosive cell proliferation, intense resistance to cell apoptosis and widespread infiltration throughout the brain. Despite multimodal therapies such as surgery, radiotherapy and chemotherapy, the median survival of glioblastoma (GBM) is less than 1 year [1]. Novel therapeutic approaches are needed to improve long-term survival for this cancer. Recent advances in our understanding of the altered genes and pathways in malignant gliomas offer the opportunities for new therapeutic strategy based on targeting essential molecular mechanisms.

MicroRNAs (miRNAs) are small, non-protein-coding, single-stranded RNAs that regulate target genes expression post-transcriptionally in normal tissues and cancers. miRNAs bind to the 3’ UTR of target gene and lead repression or degradation of the transcript through imperfect or complete complementarity. The partial complementarity allows miRNAs to target 3’ UTR of multiple genes [2-4], and growing evidences indicate that miRNAs are involved in a range of processes including cellular development, apoptosis and disease in human [5-8]. A recent study demonstrates that more than 50% of miRNA genes are present in cancer associated genomic regions or fragile sites [9], suggesting that miRNAs may play important roles in cancer pathogenesis, diagnosis and progression [10-12].

The EGFR, a member of the erbB receptor family which widely express in all tissues and regulate the normal cellular processes, is frequently amplified in a variety of human malignancies, especially glioblastoma multiforme [13-16]. In GBM patients, EGFR amplification was a significant predictor for poor survival prognosis [17], thus EGFR and its downstream members are ideal therapeutic targets.
MiR-7 is an intrinsic miRNA that resides in the first intron of heterogeneous ribonucleoprotein K gene on chromosome 9 and conserved across all species. In previous reports, it has been reported that IGF1R (insulin-like growth factor 1 receptor) is targeted by miR-7 in tongue squamous cell carcinoma cells which in turn resulted in a reduction of cell proliferation and an enhanced apoptotic rate. And PAK1, a serine/threonine kinase that plays a pivotal role in cell migration and invasion, is also targeted by miR-7. The recent findings show the relationships between miR-7 and the proto-oncogenes (e.g. PAK1, RAF1 and EGFR) suggest that miR-7 plays major roles in tumorigenesis [18-21].

It is well known that the mature miR-7 expression dramatically decreased in glioma compared with adjacent normal brain tissues [21, 22]. Excitingly, the EGFR expression was negatively regulated by miR-7 [21], and deficiency of miR-7 function in glioma cells cause downstream molecules switch on or off, which in turn affects glioma cells vitality and disease process.

To date, most researches have been done to explore the functional roles for miR-7 in various cells in vitro, few are but focused on its treatment value in vivo. In our current study, we firstly presented the demonstration that systemic delivery of plasmid-mediated miR-7 encapsulated in cationic liposome significantly inhibited glioma cell growth and metastasis in vivo. These findings suggested that miR-7 should be a potential approach for human malignant glioma therapy by targeting EGFR signaling.

Materials and methods

Plasmid construction. A 150-base pair genomic fragment spanning the hsa-miR-7 was inserted into pGenesil-2.1 expression vector (Genesil Biotechnology Company, Wuhan, China) at its BamHI / HindIII sites to express miR-7. All the sequences were confirmed by DNA sequencing. The empty expression plasmid named KB was designated as control. Plasmids were prepared using Endo-free Plasmid Giga kit (Qiagen, Germantown, MD, USA) according to the manufacturer’s direction.

Cell culture and transfection. Human glioma cell lines U-87MG and U-118MG were acquired from the American Type Culture Collection (ATCC, Rockville, MD, USA), and U251 was obtained from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS at 37°C under 5% CO₂ in atmosphere. Plasmids miR-7 and KB (2 μg each) were transfected into cells seeded in six-well plates by Fugene HD Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer’s instruction.

In vitro proliferation analysis. Cell proliferation was analyzed by colony formation assay and cell cycle analysis. For colony formation assay, cells transfected with miR-7, KB or untreated were trypsinized and seeded at 500 cells/well in six-well plates. For 8-11 days later, cells were stained with crys-
liposome/100 µl total volume per mouse). The resulting complexes were incubated at room temperature for 30 min before intravenous injection in vivo.

**Animal experiments.** All animal protocols were approved by the Institutional Animal Care and Treatment Committee of Sichuan University (Chengdu, Sichuan, China). U-87MG (5×10⁶ cells) and U251 (3×10⁶ cells) in 0.1 ml serum-free DMEM medium were inoculated subcutaneously into the right flank of each female athymic mouse (3-4 weeks old), respectively. When the size of tumor reached around 100 mm³, the animals were randomly divided into three groups (5 mice per group): 5% GS, KB and miR-7. Tumor-bearing mice were treated intravenously through the tail vein every two days for 4 weeks (U-87MG) or 5 weeks (U251), and the tumor volume was measured with a caliper every 3 days and calculated according to the formula: Tumor volume = length×width²/2. Animals were sacrificed three days after the last injection, and solid tumor tissues were removed and weighed. Part of the tumor tissues was fixed in 4% paraformaldehyde and embedded in paraffin, and the rest was immediately frozen.

Since U251 xenograft model can spontaneous formed pulmonary and lymph node metastasis after 4-5 weeks inoculation, lungs and lymph node tissues of the animals were excised. Tumor metastases on lung surfaces were counted under a dissecting microscope and photographed after be fixed in 4% paraformaldehyde solution, subsequently the lung and lymph node tissues were paraffin embedded and sectioned for further pathologic analysis.

**Detection of apoptosis.** Cell apoptosis in situ was determined using TUNEL assay kit (Promega, San Luis Obispo, CA, USA) according to the manufacturer’s protocol. The number
of TUNEL-positive cells which represented apoptotic cells were counted under a fluorescence microscopy (× 200) in six randomly selected fields. The apoptotic index was defined as follows: apoptotic index (%) = 100 × apoptotic cells/total tumor cells.

Quantitative real time PCR. As described previously [24], total RNA was extracted from glioma cancer cells and xenograft tumors using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and quantitative real time PCR (qRT-PCR) analysis was performed to detect the expression of candidate miRNAs with the TaqMan MicroRNA assays kit (Applied Biosystems, Foster City, CA, USA).

Statistical analysis. All statistical analyses were performed using SPSS 13.0. Data were expressed as the mean ± SD and were analyzed statistically using one-way ANOVA. Differences were considered statistically significant if \( P < 0.05 \).

Results

Effect of miR-7 on human glioma cell lines. To detect the functional effects of miR-7 on human glioma cell viability in vitro, cell proliferation analysis, flow cytometric analysis, cell migration and invasion analysis was performed. According to the results of sequent experiments, a decrease in the number of cell colonies, an increased percentage of cells in G0 / G1 phase (Fig. 1A, 1B), an elevation of sub-G1 phase cell population (Fig. 1C), a reduced area of cell migration and depressed cell invasion ability (Fig. 2A, 2B) were distinctly observed in group treated with miR-7. Hoechst 33258 staining was also performed to assess cell apoptosis by morphological changes. More condensed nucleus which were characteristic of apoptosis, were captured by fluorescence microscope in miR-7 treated group (Fig. 1D), whereas there were no significant changes in control groups. These results indicated that glioma cells transfected with miR-7 in vitro could evidently inhibit cell growth, cell migration, invasion, meanwhile induce cell apoptosis.

Regulation of miR-7 on EGFR signaling pathways. EGFR overexpression leads to the activation of various downstream signaling effectors that are responsible for important cellular processes, such as proliferation, apoptosis, cell migration and invasion. To investigate whether miR-7 inhibited EGFR and its downstream signaling members, we screened some essential kinases involved in EGFR signaling pathway, and the results were consistent with the effect discussed above. MiR-7 significantly suppressed the phosphorylation of Akt,
ERK and Stat3, as well as MMP-2, MMP-9, Survivin and PCNA (Fig. 3A, 3B), which suggested that miR-7 exerted its antitumor function by direct targeting EGFR on the surface of glioma cells and further antagonizing EGFR-mediated downstream signaling cascade (Fig. 3C).

Effect of miR-7 on tumor progression in vivo. To further investigate the effects of miR-7 on tumor progression in vivo, subcutaneous xenografts of glioma were established and treated as described in the Materials and Methods. Compared with control groups, primary tumor growth were sharply suppressed and mean tumor weight were evidently reduced in miR-7 group (Fig. 4A, 4B; \(P < 0.01\)); conversely, no significant differences were observed between the two control groups (\(P > 0.05\)). Moreover, to explore the effects of miR-7 on cells apoptosis in vivo, TUNEL analysis was carried out in tumor sections. In coincidence with the results in vitro, TUNEL-positive nuclei (with green staining) were increased significantly in mice treated with miR-7 (Fig. 4C, \(P < 0.01\)).

Furthermore, to verify the antitumor effects caused by miR-7 treatment, we detected the mature miR-7 in glioma subcutaneous xenografts by qRT-PCR. Compared with control groups, the expression of miR-7 sharply increased in group treated with miR-7 (Fig. 5B, \(P < 0.05\)), whereas there were no significant differences between the two control groups (\(P > 0.05\)). And the results were consistent with the detection in vitro (Fig. 5A). Meanwhile, the EGFR and PCNA expression in tumors treated with miR-7 were obviously down-regulated (Fig. 5C, D).

In addition, to evaluate potential drug toxicity on mice during the treatment, we continuously evaluated relevant indexes such as weight, appetite, diarrhea and behavior. No significant side reactions were found in gross measures. Moreover, no pathological changes in heart, liver, spleen, lungs, or kidneys of treated mice were detected by microscopic examination (data not shown).

Effect of miR-7 on U251 lung and lymph node metastases. Untreatable metastasis was the main cause of mortality in pa-
patients with cancer. As in our model, we used the U251 human metastatic glioma cells, which had a high metastatic potential and can metastasize to the lung approximately 4 weeks after inoculation [25]. Meanwhile, we found the lymph nodes were swollen. When the tumor xenografts volume reached 3,000 mm$^3$ in our present study, mice were sacrificed and the metastatic nodules on lung surface were counted under a dissecting microscope. A dramatic decrease in the number of tumor metastases was observed in miR-7 treated group compared with others groups (Fig. 6A, C; $P < 0.01$). H&E staining of lung and lymph node tissue sections also showed that the miR-7 group had much fewer and smaller metastases than controls (Fig. 6B, D; $P < 0.01$).

**Discussion**

Cancer cells frequently contain multiple genetic and epigenetic abnormalities. Malignant tumors are generally heterogeneous and caused by the accumulation of genetic alterations in oncogenes and tumor suppressor genes. Despite this complexity, numerous studies indicate that the tumor progression can also be driven by the activity of specific
oncogene [26, 27]. Over the years, based on many molecular genetic and histopathological researches, it is known that GBM is a highly malignant tumor with remarkable genomic heterogeneity, and the EGFR is one of the most deregulated components of human GBM [28, 29]. Consequently, it promoted the development of new anti-EGFR therapeutics such as monoclonal antibodies and tyrosine kinase inhibitors. However, the clinical efficacy of EGFR inhibitor is ultimately limited by the development of acquired drug resistance [30, 31]. Recently, many miRNAs are considered to function as tumor suppressors or oncogenes in human tumors (10, 32, 33). Factually, it is the promising strategy of using miRNAs for anticancer therapy. Here, we have demonstrated miR-7 as a key tumor suppressor in glioma that functions as a negative regulator of EGFR signaling.

In previous reports, miR-7 has been characterized as tumor suppressors in many human cancer cells and plays important roles in cell proliferation, apoptosis and migration in vitro [21, 34]. In our present study, we performed mainly further investigation on the therapeutic effect of miR-7 for human glioma in vivo. We used a plasmid-mediated miRNA expression system to express the miR-7 and quantitatively analyzed the anti-tumor effects in vivo. Systematic delivery of miR-7/liposome complex to subcutaneous glioma xenografts induced significant suppression on primary tumor growth (approximately 40% in tumor volume and tumor weight) and metastatic nodules (60% suppression ratio in lung metastases and 80% suppression ratio in lymph node metastases, respectively). These data provided convincing evidences that miR-7 could inhibit glioma xenografts growth and metastasis in vivo and suggested miR-7 might offer a clinically feasible approach for glioma therapy.

Mechanisms of the potent antitumor and anti-metastasis efficacy remain to be fully elucidated. Overexpression of EGFR in human malignancies induces cascades of downstream signaling, such as activation of MAPK/ERK, PI3K/Akt and Jak/Stat, which mediate cell survival, proliferation, apoptosis, invasion, tumor progression and metastasis [13, 33, 34]. MiR-7 targets several proto-oncogenes, including RAF1, IGF1R and PAK1 (18-20). Thus, it is likely that miR-7 contributes to tumor progression through multiple genes of multiple pathways. However, our present study demonstrated that miR-7-mediated down-regulation of EGFR led a reduction in cell proliferation, invasion and an enhanced apoptotic rate.

Delivery is still the therapeutic bottleneck in vivo. In this study, we selected the delivery system of DNA-Cationic liposome complexes. The selection of cationic liposome rooted mainly in the parameters of biodegradation, biodistribution, and toxicity [23]. And the validity of DNA-Cationic liposome complexes delivery was supported by previous studies using

![Figure 5](image-url)
cationic liposome to deliver tumor suppressor genes and inhibit tumor growth [36]. Furthermore, FUS1-Cationic liposome complex had been advanced into phase I clinical trial for treatment of NSCLC [37]. Therefore, the safe and efficient delivery of the cationic liposome vector appeared to be attractive.

In summary, to our knowledge, it is the first demonstration that plasmid-based miR-7 could safely and efficiently suppress glioma xenograft growth and metastases in vivo. The results are encouraging since we regard miR-7 as considerable therapeutic potential suppressor gene for glioma therapy.

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References


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