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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Let-7 microRNA inhibits the proliferation of human glioblastoma cells

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs comprising 21–23 nucleotides that regulate gene expression by transcriptionally repressing their complementary mRNAs. In particular, let-7 miRNA has been postulated to function as a tumor suppressor in various cancer cells, but not yet in glioblastoma. In this study, we investigated the anti-tumorigenic effect of let-7 miRNA in glioblastoma cells. Human glioblastoma cells (U251 or U87 cells) were transfected with let-7 miRNA and assayed for in-vitro proliferation, migration, and in-vivo tumor formation. Transfection of let-7 miRNA reduced expression of pan-RAS, N-RAS, and K-RAS in the glioblastoma cells. Let-7 miRNA also reduced the in-vitro proliferation and migration of the cells, and reduced the sizes of the tumors produced after transplantation into nude mice. However, let-7 miRNA exerted no effect on the proliferation of normal human astrocytes. These results indicate that let-7 miRNA has an anti-tumorigenic effect on glioblastoma cells, and suggest possible use of let-7 miRNA for treating glioblastoma.

Keywords Let-7 · miRNA · Glioblastoma · Ras · Proliferation

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Introduction

Glioblastoma is the most common primary malignant brain tumor found in adults, and patients respond poorly to chemotherapy, except for a small subgroup that benefits from administration of temozolomide or epidermal growth factor receptor (EGFR) kinase inhibitor [1]. MicroRNAs (miRNA) are small, noncoding RNAs with 21–23 nucleotides that regulate gene expression by repressing transcription or degrading target mRNA [2]. In cancer cells, miRNAs can function as tumor suppressors or oncogenes, and, thus, can regulate tumor development and prognosis [3].

In particular, let-7 miRNA has been postulated to function as a tumor suppressor by silencing RAS and high mobility group AT-hook 2 (HMGA2) [4, 5]. Let-7 was originally identified in *C. elegans* as a regulator of developmental timing and cellular proliferation [6], and several let-7 congeners (let-7a–k) have since been identified. Let-7 has been reported to be reduced in human lung cancer [7], colon cancer [8], and melanoma [9], and the delivery of let-7 has been found to reduce cancer cell proliferation and tumorigenicity [10, 11]. On the other hand, the oncogene Ras is a member of the small GTPase superfamily and the signaling mediator of ephrin receptors, and has been implicated in cancer cell physiology, particularly in cell proliferation, adhesion, and migration [12]. In glioblastoma, Ras signaling regulates glioblastoma cell growth, survival, and invasion [13], and Ras inhibition reduces tumorigenicity and the migration potential of glioblastoma cells [14, 15]. The above-mentioned results suggest that let-7 miRNA is likely to reduce glioblastoma growth and cellular migration via Ras inhibition. Thus, in this study, we investigated the effect of let-7 miRNA delivery on the proliferation, migration, and tumorigenicity of glioblastoma cells.

Materials and methods

Cell culture and miRNA transfection

U251 and U87 human glioblastoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Gibco, Invitrogen, Carlsbad, CA, USA). Normal human astrocytes (Lonza, Basel, Switzerland) were cultured in Clonetics AGM Bulletkit (Lonza). Cells were maintained in a humidified 5% CO_2 atmosphere at 37°C. Prepared cells were transfected in 24-well plates with pre-let-7 g (Ambion, Applied Biosystems, Foster City, CA, USA), a negative control miRNA (Ambion), or a cy3-labeled negative control miRNA (Ambion), using Lipofectamine 2000 (Invitrogen). Of the known let-7 family members, let-7 g was selected for this study on the basis of its proved action on lung cancer cells [11]; the mature sequence of let-7g is 5′-uga ggu agu agu uug uac agu u-3′.

Immunocytochemistry

Cell cultures were processed for immunocytochemistry, as previously described [16]. Cells (5×10^3) were plated on 12-mm round Aclar plastic coverslips coated with 10 μg/ml polylysine and housed in 35 mm dishes. Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.5) for 40 min, washed with PBS, incubated for 2 h with blocking buffer (2% horse serum/1% BSA/0.1% Triton X-100 in PBS, pH 7.5), and incubated with primary antibodies overnight at 4°C. Anti-human Pan-RAS (Santa Cruz Biotechnology, Santa Cruz, CA, USA), N-RAS (Santa Cruz Biotechnology), and K-RAS antibodies (Santa Cruz Biotechnology) were used as primary antibodies. The cells were then incubated for 1 h at room temperature with secondary antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (1:300; Jackson Immunoresearch, West Grove, PA, USA) was used as secondary antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) after each experiment. The optical densities (OD) of RAS expression were analyzed quantitatively using an image-analysis system (Image Pro-Plus, Media Cybernetics, Bethesda, MD, USA). Five color images were acquired randomly per experiment group using a digital camera connected to a fluorescence microscope, and transformed to gray-scale images. Mean light transmission was measured on a scale of 0–256 relative units and averaged.

WST-1 assays

Cell proliferations were determined by WST-1 assay agent (5 mmol/l, 1:9; Roche, Basel, Switzerland), according to the manufacturer's instructions. Higher values indicate greater cell numbers. Absorbances were measured at 450 nm (reference at 630 nm) using a Multiskan JX microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Experiments were performed in quadruplicate.

The in-vitro scratch migration assay

Glioblastoma cells were seeded into 24-well plates and cultured to subconfluence. Cell monolayers were then scratched with a 1,000-μl plastic pipette tip to create a cell-free area (a "wound") approximately 2 mm wide [16]. Wounded monolayers were washed twice with culture medium to remove loose cell debris, and wounds were photographed under a phase-contrast microscope. Cell migration was quantified after 24 h by counting cells that had migrated into the wounded areas, and the relative cell migration (%) was calculated as [the number of let-7-treated cells that migrated into wounds/the number of control-miRNA-treated cells that migrated into wounds] × 100. Experiments were performed in triplicate.

In-vivo assays

Six-week-old male nude mice (Orient, Seoul, South Korea; $n = 4$) were used as hosts for glioblastoma xenografts. Briefly, let-7-transfected U251 or control U251 cells were harvested, washed twice, resuspended in cold PBS at 5×10^6/100 μL, and injected subcutaneously into flanks using a 25-gauge needle ($n = 4$ tumors per group). The animals were kept under pathogen-free conditions and tumors were removed 14 days after transplantation and weighed. All experiments on animals were approved by the ethics committee at our institution.

Statistical analysis

Results are presented as means ± SD. The Mann–Whitney U test was used to determine the significance of inter-group differences. SPSS ver. 15.0 (SPSS, Chicago, IL, USA) was used for the analyses, and two-tailed P values of <0.05 were considered significant.

Results

Let-7 miRNA transfection reduced RAS levels in vitro

In a preliminary study on the transfection of Cy3-labeled-negative control miRNA in U251 glioblastoma cells,

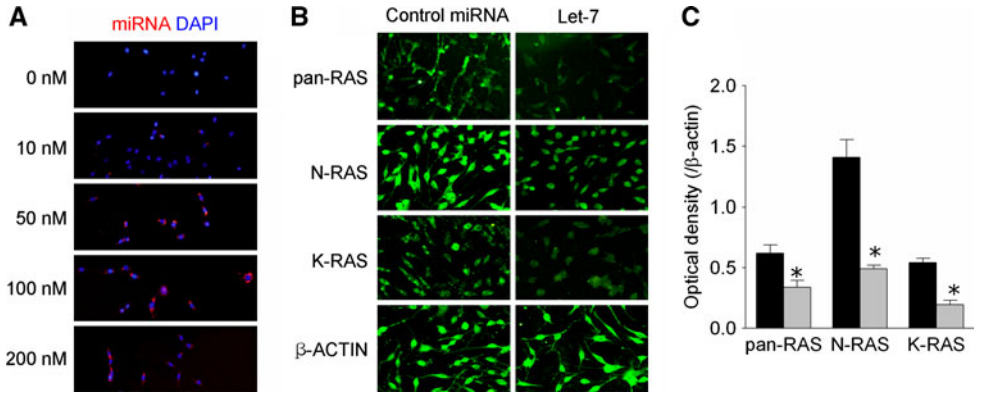

Fig. 1 Transfection of let-7 and RAS levels. **a** Cy3-labeled-control miRNA was tested for transfection efficiency in U251 glioblastoma cells at concentrations of 0–200 nM, and 100 nM was selected for further studies. **b** Let-7 miRNA-transfected U251 glioblastoma cells showed lower levels of pan-RAS, N-RAS, and K-RAS than cells transfected with control miRNA (representative photographs). **c** Optical density analysis of their immunofluorescence confirmed these results. $^*P < 0.05$ versus the control

cy3-immunofluorescence was maximum at 100 nM concentration, and higher concentrations of control miRNA caused cell deaths (Fig. 1a). On the basis of this result, we used 100 nM miRNA for further transfection experiments.

Control or let-7 miRNAs were transfected into U251 cells, and RAS protein levels were evaluated at 48 h post-transfection. Let-7 miRNA transfection reduced the expressions of pan-RAS (relative optical densities versus β-actin; control $= 0.62 \pm 0.12$, let-7 $= 0.33 \pm 0.10$), N-RAS (control $= 1.41 \pm 0.25$, let-7 $= 0.49 \pm 0.05$), and K-RAS (control $= 0.54 \pm 0.06$, let-7 $= 0.19 \pm 0.07$) compared with the control miRNA transfection (all $P < 0.05$; Fig. 1b, c).

Let-7 miRNA reduced the in-vitro proliferation and migration of glioblastoma cells

We then investigated the effect of let-7 miRNA on glioblastoma cell proliferation. At 24 h post-transfection, the cellular densities of let-7-transfected U251 cells were slightly lower than those of cells transfected with control miRNA (Fig. 2a). At 48 h, WST-1 assays confirmed significantly lower cell numbers in the let-7-transfected U251 cells compared with the control miRNA-transfected U251 cells (control miRNA $= 1.00 \pm 0.16$, let-7 $= 0.80 \pm 0.15$, $^*P < 0.05$, relative levels) (Fig. 2b). In experiments using U87 glioblastoma cells, let-7 transfection reduced the cell proliferation again (control miRNA $= 1.00 \pm 0.21$, let-7 $= 0.83 \pm 0.11$, $^*P < 0.05$, relative levels), confirming the inhibitory effect of let-7 on glioblastoma cell proliferation in the second cell line (Fig. 2b). In contrast, human normal astrocytes transfected with let-7 showed no significant reduction of cell proliferation compared with the cells transfected with the control miRNA (control miRNA $= 1.00 \pm 0.14$, let-7 $= 1.06 \pm 0.21$, $^*P = 0.667$, relative levels) (Fig. 2b), suggesting that let-7 miRNA has a different anti-proliferative effect on glioblastoma cells and normal astrocytes.

Migration, and invasion of normal tissue, are important hallmarks of glioblastoma cells [17]. In the scratch migration assay, let-7 transfection reduced cellular migration of U251 cells over scratch wounds compared with the control miRNA transfection (control miRNA $= 100 \pm 12\%$, let-7 $= 35 \pm 13\%$, $P < 0.01$) (Fig. 2c, d).

Let-7 miRNA attenuated in vivo tumor formation

U251 cells were transfected with control miRNA or let-7, and inoculated subcutaneously into nude mice. Tumors were evaluated 14 days after transplantation (Fig. 3a). Tumor weights produced by glioblastoma cells treated with let-7 were lighter that those produced by cells treated with control-miRNA (let-7 $= 60 \pm 44$ mg, control miRNA $= 233 \pm 86$ mg; $^*P < 0.05$) (Fig. 3b), indicating that let-7 attenuated glioblastoma cell proliferation in vivo.

Discussion

In this study, we investigated the anti-tumorigenic effects of let-7 miRNA transfection in glioblastoma cells by quantifying cellular proliferation and migration. Our in-vitro and in-vivo results suggest that let-7 has anti-tumorigenic and anti-migratory effects on glioblastoma cells, and that these may be because of the reduced expressions of RAS.

Recent studies have revealed that miRNAs play key roles in the pathophysiology of glioblastoma [18]. In particular, miR-21 is aberrantly over-expressed in glioblastoma cells [19–21] and involves in migration, apoptosis, invasion, and growth of the cells [21–23]. miR-21 targets multiple components of p53, transforming growth factor-beta (TGF-beta), and mitochondrial apoptosis tumor-suppressive

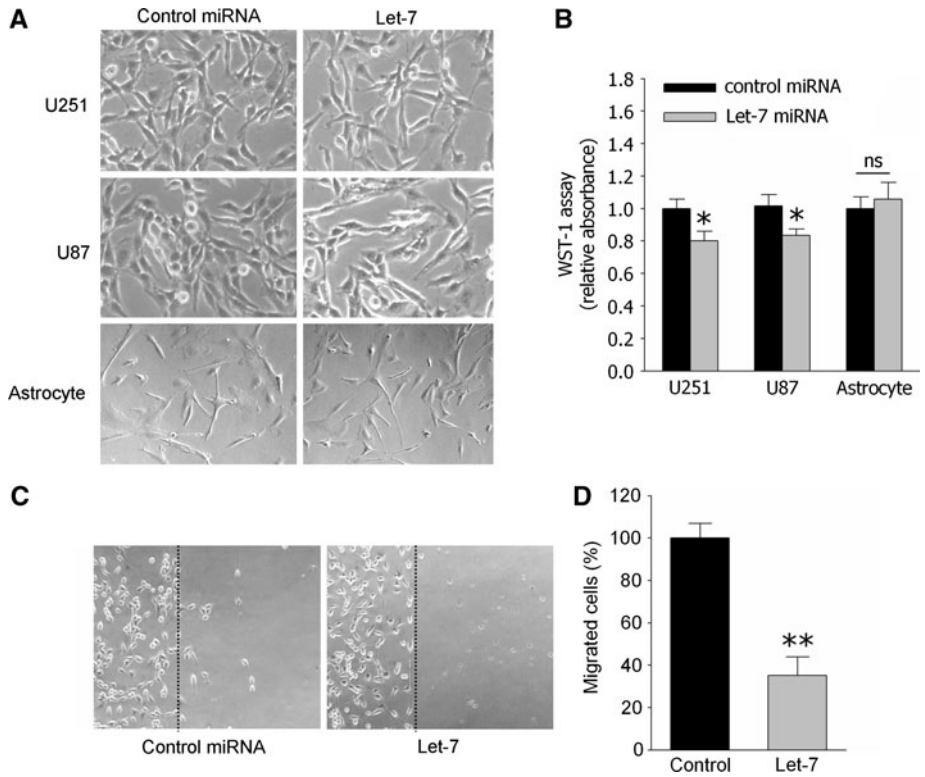

Fig. 2 In-vitro inhibition of proliferation and migration by let-7 transfection. **a** The cellular densities of cultures of U251 and U87 glioblastoma cells transfected with let-7 were slightly lower than those of control transfected cells 24 h after transfection (representative photograph). However, the cellular densities of astrocytes were similar for control and let-7 transfections. **b** In the WST-1 assay 48 h after transfection, cell numbers were lower for let-7-transfected U251 and U87 glioblastoma cells than for control miRNA transfection, whereas cell numbers for let-7-transfected astrocytes were no different from those for control miRNA transfection. In scratch migration assays (**c**, representative photographs), let-7 transfection reduced the number of U251 cells that migrated over "wounds" compared with control miRNA transfection (**d**). $*P < 0.05$ and $**P < 0.01$ versus the control. *ns*, no significant difference

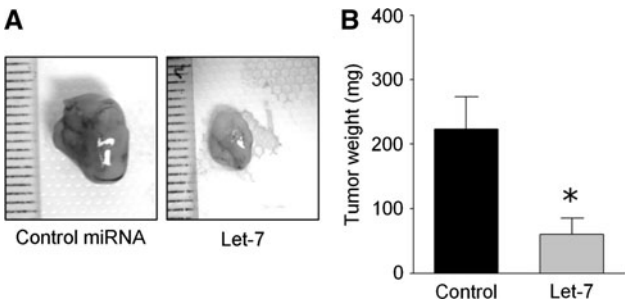

Fig. 3 let-7 attenuated in-vivo tumor formation by glioblastoma cells. **a** U251 glioblastoma cells transfected with control miRNA or let-7 were inoculated subcutaneously into the flanks of nude mice, and tumors were evaluated 14 days later. **b** Tumors from let-7-transfected cells were lighter than those from control-miRNA transfected cells. $*P < 0.05$ compared with control

pathways [20], and promotes glioblastoma invasion by targeting matrix metalloproteinase regulators [23]. Thus, knockdown of miR-21 with antagomir increases glioblastoma apoptosis [19], and reduces migratory and invasion ability of the cells, suggesting therapeutic implications [23]. In addition, diagnostic and chemotherapeutic implications of miR-21 in glioblastoma have also been suggested [24, 25]. Accordingly, whether let-7 miRNA affects the expression of miR-21 is an interesting issue, and the mechanism of how let-7 miRNA inhibits glioblastoma cell proliferation should be investigated further considering the effect of other miRNAs, for example miR-21.

There have been reports of other miRNAs implicated in glioblastoma cells. miR-124 and miR-137 both induce differentiation of glioblastoma stem cells [26], and miR-128 inhibits the proliferation of glioblastoma cells by targeting Bmi-1 [27]. miR-146b inhibits migration and invasion of the cells by downregulating matrix metalloproteinases-16 [28]. miR-7 and miR-181 are downregulated in glioblastoma [29, 30], and miR-221/222 is overexpressed, which downregulates the p27(kip1) tumor suppressor [31]. Thus, various miRNA dysregulations are involved in the pathogenesis and cellular physiology of glioblastoma cells. In lung cancer, RAS is regulated by the let-7 miRNA family [4]. Therefore, our study implies that let-7 miRNA, which is known to suppress other cancer cells also, should be viewed as one of the miRNA candidates for controlling glioblastoma cell proliferation and tumorigenicity.

miRNAs regulate gene expression by translational repression and by degradation of target mRNAs, and a single miRNA can repress the production of hundreds of proteins [32]. Nevertheless, let-7 miRNA has been known to target Ras mRNA directly [4], and to repress the activation of downstream mitogen-activated protein kinase by reducing p-ERK [33]. In our study, let-7 transfection resulted in no change in hemoglobin concentration or in expression of vascular endothelial growth factor in the xenografted tumors (data not shown), suggesting that the anti-proliferative effect is more important than the anti-angiogenic effect after let-7 transfection. However, let-7 miRNA also affects multiple genes involved in the cell cycle and represses cell division directly and indirectly [34]. Accordingly, the anti-proliferative effect of let-7 is likely to be associated with multiple genes and pathways.

As shown by a previous microarray study [21, 35], let-7 expression is not downregulated in human glioblastoma tissues and cell lines. Accordingly, the transfection of let-7 miRNA does not correct a let-7 deficiency, and could result in overexpression of let-7. However, the innate down-regulation of let-7 miRNA in target tumors is not always required for let-7 delivery to achieve an anti-tumorigenic effect, as is shown by our results and those of others [36]. Low levels of let-7 or polymorphisms in 3′ untranslated region of K-RAS predict a poor outcome in various cancers [7, 37]. In addition, reduced expression of let-7 miRNA families is associated with low responsiveness to a number of chemotherapeutic agents [38]. Overexpression of let-7 miRNA creates a radiosensitive state of lung cancer cells [39]. Accordingly, variation of let-7 expression is likely to be associated with the prognosis or therapeutic responses of glioblastoma, which warrants further studies.

A small number of viral and non-viral delivery methods have been developed that may be suitable for delivery of miRNA to glioblastoma, and in particular, antagomirs have been used to target specific miRNAs in vivo [18]. As our results have shown, let-7 miRNA has different anti-proliferative effects on glioblastoma cells and astrocytes. However, the transfection of let-7 miRNA into human fibroblasts resulted in reduced cell numbers and increased G2/M cell cycle phases [40]. Thus, it is still important to deliver let-7 miRNA specifically to glioblastoma cells. We suggest that further studies should be conducted to devise a method of delivering let-7 to glioblastoma in vivo, and to design stable chemotherapeutic agents that mimic let-7 miRNA.

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