Abstract

MicroRNA-203 modulates the radiosensitivity of human malignant glioma cells

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Purpose: We investigated whether or not miR-203 could modulate the radiosensitivity of glioblastoma (GBM) cells and which target gene(s) could be involved.

Materials and Methods: Three human malignant glioma (MG) cell lines and normal human astrocytes were transfected with control microRNA, pre-miR-203, or antisense miR-203. RT-PCR, clonogenic assays, immunofluorescence, and invasion/migration assays were performed. To predict the target(s), bioinformatics analyses using microRNA target databases were performed.

Results: The overexpression of miR-203 increased the radiosensitivity of all the three human MG cell lines and prolonged the radiation-induced γ-H2AX foci formation. The bioinformatics analyses suggested that miR-203 could be involved in the post-transcriptional control of DNA repair, PI3K/AKT, SRC, JAK/STAT3, and the vascular signaling pathway. The western blot analysis validated that miR-

203 downregulated ATM, RAD51, SRC, PLD2, PI3K-AKT, JAK-STAT3, VEGF, HIF-1α, and MMP2. Overexpression of miR-203 inhibited the invasion and migration potentials, downregulated SLUG and Vimentin, and upregulated Claudin-1 and ZO1.

Conclusions: These data demonstrate that miR-203 potentially controls DNA damage repair via the PI3K/AKT and JAK/STAT3 pathways and may collectively contribute to the modulation of radiosensitivity in MG cells by inhibiting DNA damage repair, pro-survival signaling, and Epithelial-Mesenchymal Transition. Taken together, these findings demonstrate that miR-203 could be a target for overcoming the radioresistance of GBM.

Keywords: microRNA-203, glioblastoma, radiosensitivity

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Introduction

Despite a multimodal approach that includes maximal surgery, radiotherapy (RT), and temozolomide (TMZ), patients with glioblastoma multiforme (GBM) have a grave prognosis, with a reported median survival of 14-16 months (1). GBM's highly aggressive and infiltrative nature, intratumoral hypoxia, inherent molecular heterogeneity, and resistance to treatment have made the devastating outcome of GBM difficult to improve. To enhance the therapeutic outcome of GBM, many attempts have been made to modulate its deregulated signaling pathways. TMZ improved the survival outcome of GBM patients, but local progression predominated among the recurrences even after concurrent chemoradiotherapy with TMZ, which indicates the presence of persistent treatment resistance in the TMZ era (2). Therefore, the investigation of other biological pathways has become the novel focus of efforts to overcome GBM resistance.

MicroRNA (miRNA) plays a role in tumorigenesis and in treatment sensitivity at the post-transcriptional level in various cancer cases. miRNAs are small non-coding RNA molecules (~22 nucleotides) that bind in the 3'-untranslated region (3'-UTR) and repress mRNA translation or directly degrade mRNA (3). Using this mechanism, many studies have investigated the role of miRNA in radiosensitivity in cases of malignancy. Several miRNAs have been reported to be related to the radiosensitivity of GBM (4-6).

miR-203 is one of the cancer-associated miRNAs that show a relationship with various malignancies, including GBM. He et al. performed real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) on glioma

and normal brain tissues to determine the expression levels of miR-203 and showed that the miR-203 expression level was significantly lower in the glioma tissues than in the normal brain tissues, and that the expression level was significantly correlated with the WHO grade and survival (7). These results were replicated in other studies (8,9). The analysis of The Cancer Genome Atlas (TCGA) data showed that the miR-203 levels were suppressed in the tumor samples by 23% (9). However, to date, little is known on the molecular mechanisms of the effect of miR-203 on GBM. Moreover, whether or not miR-203 sensitizes malignant glioma (MG) cells to radiation has not been examined yet.

In this study, we investigated the effect of miR-203 on the radiosensitivity of the human MG cell line and its related molecular biological mechanisms.

Methods

Cell culture, miRNA transfection, and quantitative real-time PCR

U251, U373, and T98G MG cells and normal human astrocytes (NHA) were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37℃ atmosphere with 5% CO_2. They were plated in six-well culture plates and transfected with 50nm pre-miR-203 (mature miR-203 sequence: 5'-GUGAAAUGUUUAGGACCACUAG-3'), antisense miR-203 (Panagene, Inc.; sequence: RRRQRRKKR-OO-GTGGTCCTAAACATTTCA), or negative-control pre-miRNAs using the siPORT NeoFX™ transfection agent (Ambion, Austin, TX, USA). Assays to quantify the miRNAs were performed using the Taqman miRNA Reverse Transcription kit and the Fast Real-time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The relative fold-change of the miR-203 levels was calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to an hsa-miR-203 loading control.

Clonogenic assay

The experiment and analysis of the clonogenic assay were performed as previously described (10). The cells were irradiated with four single-radiation doses (2, 4, 6, and 8 Gy) using 6MV X-rays from a linear accelerator (Clinac 21EX, Varian Medical Systems, Palo Alto, CA, USA) at a dose rate of 2.46 Gy/min. After their incubation at 37℃ for 10 days, colonies were fixed with methanol and stained with

crystal violet, and those that contained >50 cells were counted. The survival data were fit into a linear-quadratic model using Kaleidagraph version 3.51 (Synergy Software, Reading, PA, USA). The sensitizer enhancement ratio (SER) was calculated as the ratio of the isoeffective dose at the surviving fraction of 0.5.

Bioinformatics analysis

To select the target genes of miR-203 that might modulate radiosensitivity in the MG cells, we predicted the probable target genes of miR-203 using the microRNA target databases Targetscan (http://targetscan.org/), picTar (http://pictar.mdc-berlin.de/), MiRanda (http://www.microrna.org/microrna/home.do), miRWalk (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/index.html), and miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/). A selected group of genes that were predicted to be targets in multiple databases with high frequency and that showed a possible relationship with radiosensitivity in GBM in the literatures including the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and PubMed, were chosen for validation.

Western blot analysis and immunocytochemistry

Western blot analysis and immunocytochemistry were performed as described previously (10). Briefly, cell lysates from the transfected cells were prepared through the addition of a lysis buffer (iNtRON Bio-technology, Seoul, Korea). Equal amounts of total protein were separated by 12% SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked in 5% powdered milk for an hour and

incubated with the primary antibodies overnight at 4℃, and then incubated with a peroidase-labeled goat anti-rabbit or anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for an hour at room temperature. The primary antibodies of the phosphorylated ataxia telangiectasia mutated (*p*-ATM; Ser1981, Cell Signaling Technology), and RAD51 (Merck Millipore), DNA-PK (Thr2609, Abcam), phosphorylated PI3K (*p*-PI3K; #4228), phosphorylated AKT (*p*-AKT; Ser473, Cell Signaling Technology), PLD2 (Y169, Abcam), phosphorylated SRC *(p*-SRC; Tyr416), phosphorylated JAK1 (*p*-JAK1; Tyr1022/1023), phosphorylated STAT3 (*p*-STAT3), SLUG, Vimentin (Cell Signaling Technology), ZO1 (Abcam), Claudin-1 (Cell Signaling Technology), VEGF (Abcam), MMP2, HIF1-α (Cell Signaling Technology), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for the western blot analysis after their dilution at 1:1000. For the immunocytochemistry, the cells were seeded on a chamber slide, fixed with 4% paraformaldehyde, probed with antibodies of γ-H2AX, 53BP1 (Cell Signaling Technology) and VEGF (Abcam), and incubated with the Alexa Flour 488 secondary antibody (Molecular Probes, Eugene, OR, USA). The nuclei were counterstained with DAPI (4', 6-diamidino-2-phenylindole). The slides were examined with an Axio Scope.A1 Imager fluorescent microscope. Images were captured using AxioCam MRc5 and the acquisition software Axio-Vision v.4.4 (Carl Zeiss, Gottingen, Germany).

Modified Boyden chamber migration assay

The migratory capacity of the U251 cells was assessed using Transwell with 8μm pores (Corning, Rochester, NY, USA), according to the manufacturer's instructions.

After the transfection, the cells were diluted and plated on top of Matrigel-coated invasion chambers. After 24-hour incubation, the invading cells on the lower surface were fixed with methanol, stained with 1% crystal violet, and counted.

Wound healing assay

For the wound healing assay, when the cells grew to a confluence of 90% in six-well plates (Sonic-Seal Slide; Nalge Nunc, Rochester, NY, USA), the cell monolayer was scratched using the tip of a micropipette 48 hours after its transfection. The plates were incubated, and wound healing was visualized by comparing the photographs taken 24 hours after the washing and removing the detached cells.

Vasculogenic mimicry formation

The experiment procedures using the Matrigel assay kit (BD biosciences) were performed as previously decribed (11). The cells were transfected with negative-control pre-miRNA or 50nm pre-miR-203, and plated on the Matrigel surface. Twenty-four hours after the seeding, the tube-like structures were examined with an inverted microscope.

Statistical analysis

All the experiments were performed thrice. The data were analyzed with the two-tailed unpaired Student's t-test. A value of $P < 0.05$ was considered statistically significant.

Results

miR-203-induced increase in the radiosensitivity of the MG cells

The MG cells were transfected with pre-miR-203, and their expression was detected via RT-PCR. The overexpression of miR-203 increased the radiosensitivity of the U251, U373, and T98G MG cell lines, whereas there was no change in the radiosensitivity of NHA (Figure 1). The miR-203-transfected cells were irradiated with 6 Gy and showed prolongation of the γ-H2AX and 53BP1 foci levels in the U251 cells while the anti-miR-203-transfected cells showed a low foci level compared to the control group (Figure 2).

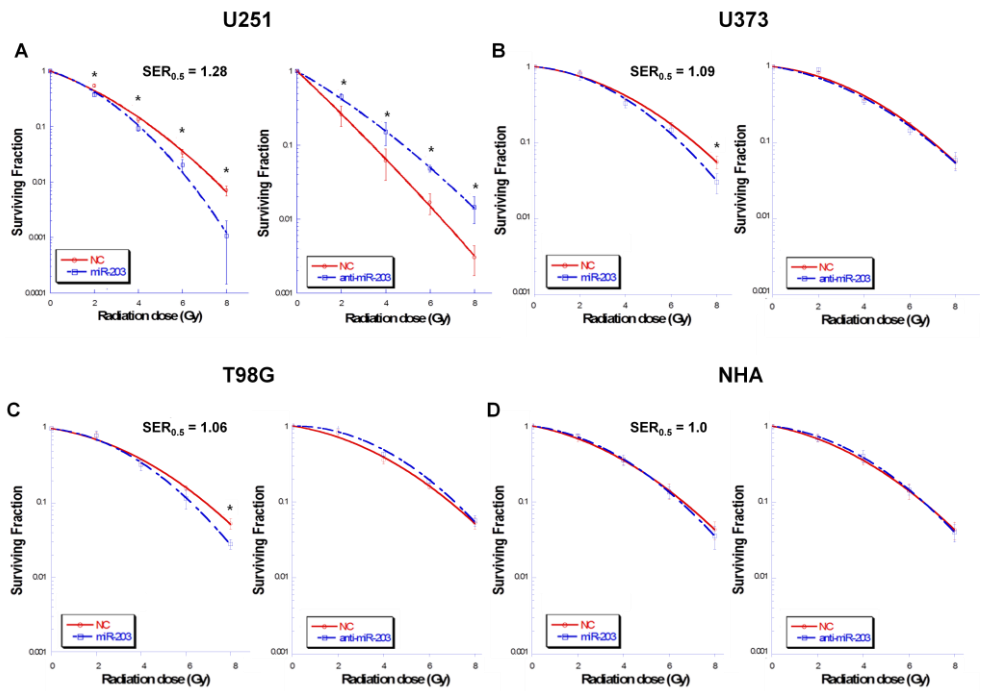

Figure 1. Clonogenic assays of U251, U373, T98G, and NHA cells transfected with pre-miR-203/control and antisense miR-203/control. A clonogenic assay was performed to determine the surviving fraction. miR-203

increased the radiosensitivity of the MG cell lines. The graph represents the mean values ± s.d. of the three experiments. (A) In an in vitro clonogenic assay, overexpression of miR-203 decreased the survival fraction of U251 MG cells after radiation compared to the control. In contrast, treatment with antisense miR-203 decreased the radiosensitivity of the MG cell lines. The radiosensitizing effect of miR-203 on MG cells was also shown on the (B) U373 and (C) T98G cell lines, whereas there was no significant change in the radiosensitivity of miR-203 on (D) NHA ($^{*}P < 0.05$; vs. control).

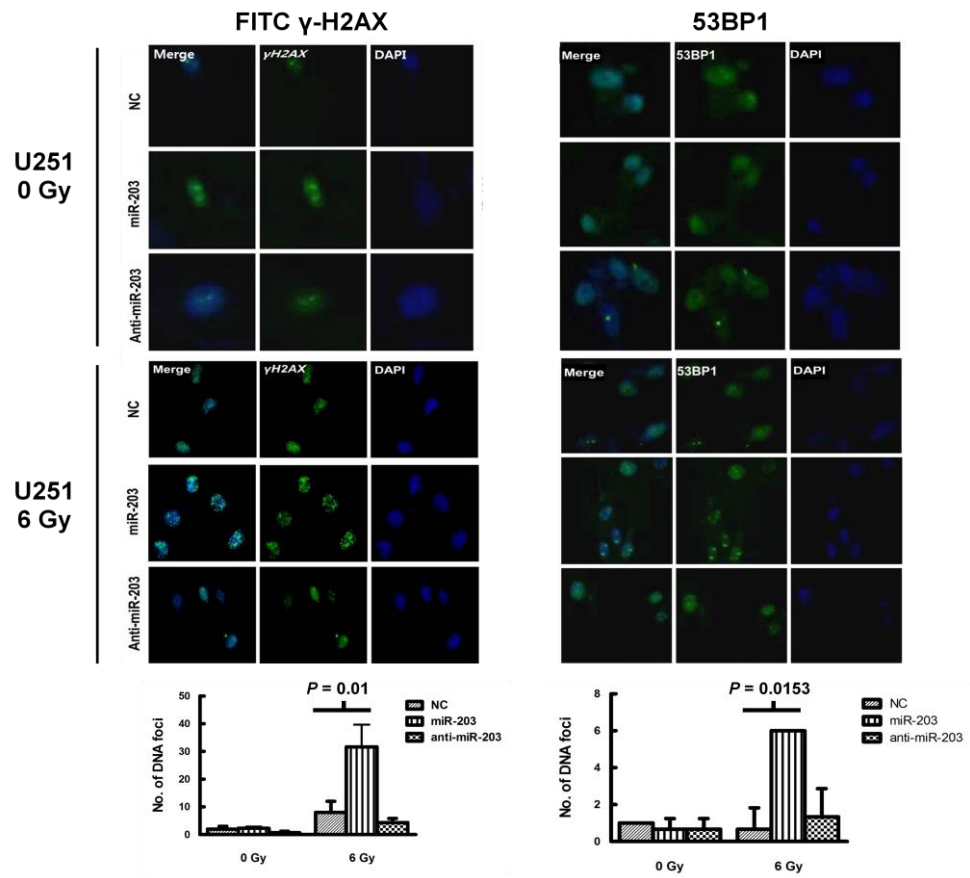

Figure 2. Representative images of γ-H2AX and 53BP1 foci formation in U251 cells. The effect of miR-203 on the repair of radiation-induced

DNA double-strand breaks (DSBs) was tested via immunofluorescence. The miR-203-transfected U251 cells were exposed to radiation, and the DNA foci representing unrepaired DSBs were stained with anti-γ-H2AX (left panel) and anti-53BP1 (right panel). The miR-203-transfected U251 cells showed persistent γ-H2AX and 53BP1 foci formation compared to the controls six hours after 6Gy irradiation.

Target prediction and validation

The major cell killing mechanism of irradiation is DNA damage, and the crucial factor of radioresistance is the DNA damage response. Therefore, we searched for DNA damage response-related genes in miRNA target prediction algorithms. Among the possible radiosensitivity-related genes in MG cells, we found that ATM, RAD51, AKT, PLD2, SRC, and JAK1 had a high possibility of being target genes of miR-203, as predicted by multiple algorithms. Based on these data, we performed western blots to elucidate the mechanism of radiosensitivity modulation by miR-203 in U251 cells.

The western blot analysis indicated that *p*-ATM and RAD51, which act in the homologous recombination (HR), were present at decreased levels in the miR-203-transfected U251 cells compared to the control (Figure 3). There was no difference in the DNA-PK level.

To investigate the mechanism of miR-203-induced radiosensitivity, we examined pathways acknowledged as linked with radiosensitivity. As shown in Figure 4, miR-203 overexpression decreased the expression of *p*-SRC, PLD2, *p*-PI3K, *p*-AKT, *p*-JAK1, and *p*-STAT3. Conversely, the expression levels of these

proteins increased when miR-203 was inhibited in the U251 cells. These results suggest that miR-203 impairs the PI3K/AKT and JAK/STAT3 pathways, which are crucial pathways of the survival and proliferation of U251 cells.

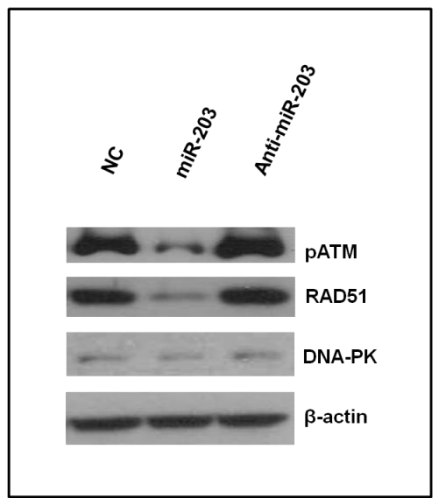

Figure 3. The western blot analysis of U251 cells (p-ATM, p-RAD51). The western blot analysis showed that *p*-ATM and RAD51 were present at decreased levels in the miR-203-transfected U251 cells compared to the control.

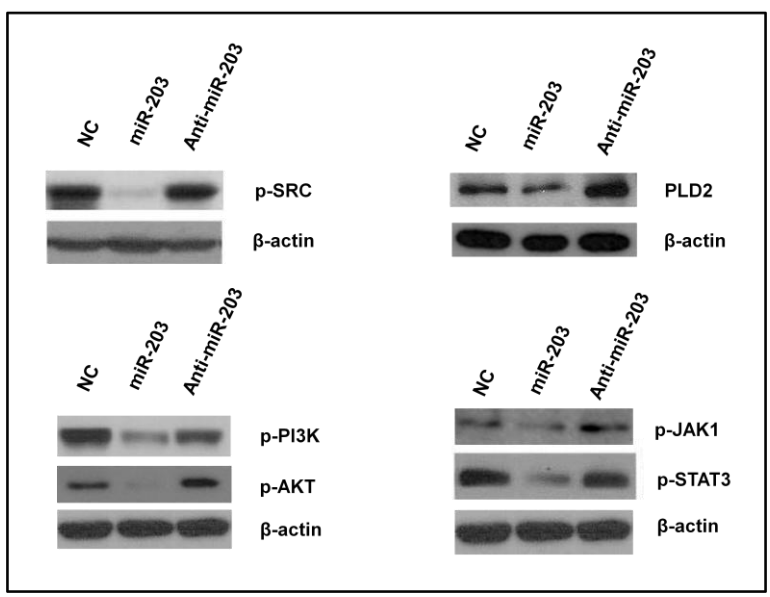

Figure 4. The western blot analysis of U251 cells (p-SRC, PLD2, p-PI3K, p-

AKT, JAK1, and p-STAT3). Overexpression of miR-203 decreased the expression of *p*-SRC, PLD2, *p*-PI3K, *p*-AKT, JAK1, and *p*-STAT3 in the western blot analysis. Conversely, the expression levels of these proteins increased when miR-203 was inhibited in their U251 cells.

miR-203-induced reversal of the Epithelial-Mesenchymal Transition (EMT)

Figure 5A shows the results of the wound healing assay and the Modified Boyden chamber assay. The cell migration significantly decreased in the miR-203-transfected U251 cells and increased in the antisense miR-203-transfected cells ($P = 0.015$). The invasion potential was significantly inhibited in the miR-203-transfected U251 cells, whereas the antisense miR-203-treated cells showed increased invasion potential ($P < 0.001$, Figure 5B). The miR-203-transfected U251 cells upregulated the epithelial markers ZO-1 and Claudin-1, and downregulated the mesenchymal markers Vimentin and Slug (Figure 5C). These findings indicate that ectopic overexpression of miR-203 reverses the EMT process.

The VM assay represents the *de novo* tumor perfusion pathway independent of endothelial cell angiogenesis. We performed this assay to investigate whether or not miR-203 regulates VM. We found that VM was significantly reduced in the miR-203-transfected cells (Figure 6A). The miR-203 transfection decreased the expression of VEGF, MMP2, and HIF1-α (Figure 6B and C).

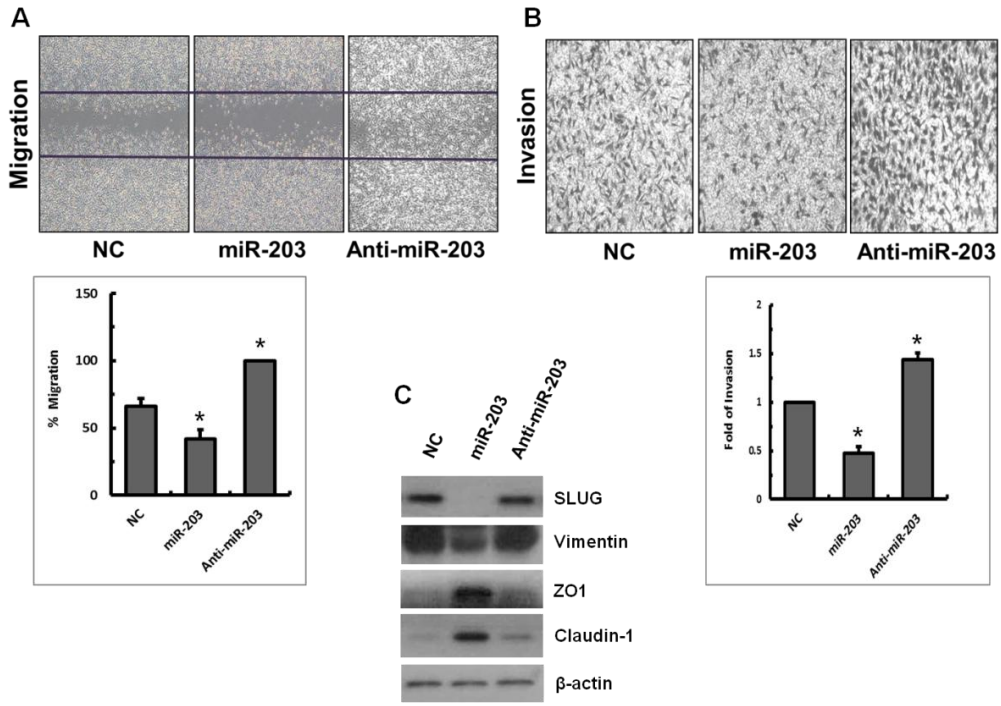

Figure 5. Representative images of (A) migration assay, (B) invasion assay, and (C) Immunoblotting of EMT-related proteins. The effect of miR-203 on cell migration and invasion was evaluated using a wound healing assay and a Modified Boyden chamber assay. (A, B) Overexpression of miR-203 resulted in the inhibition of (A) migration and (B) invasion, whereas the antisense miR-203-transfected cells showed increases in migration and invasion. Stained cells were in representative fields (100x magnification). The number of migrated (left panel) and invaded cells (right panel) were counted after 24 hours and shown as histograms. The graph represents mean values ± s.d. of three experiments; the asterisk indicates significant differences by the Student's t-test ($P < 0.05$). (C) Immunoblotting of EMT-related proteins showed that combining ectopic miR-203 expression with radiation increased the epithelial markers ZO-1 and Claudin-1 with a concomitant decrease in the mesenchymal markers Vimentin and Slug.

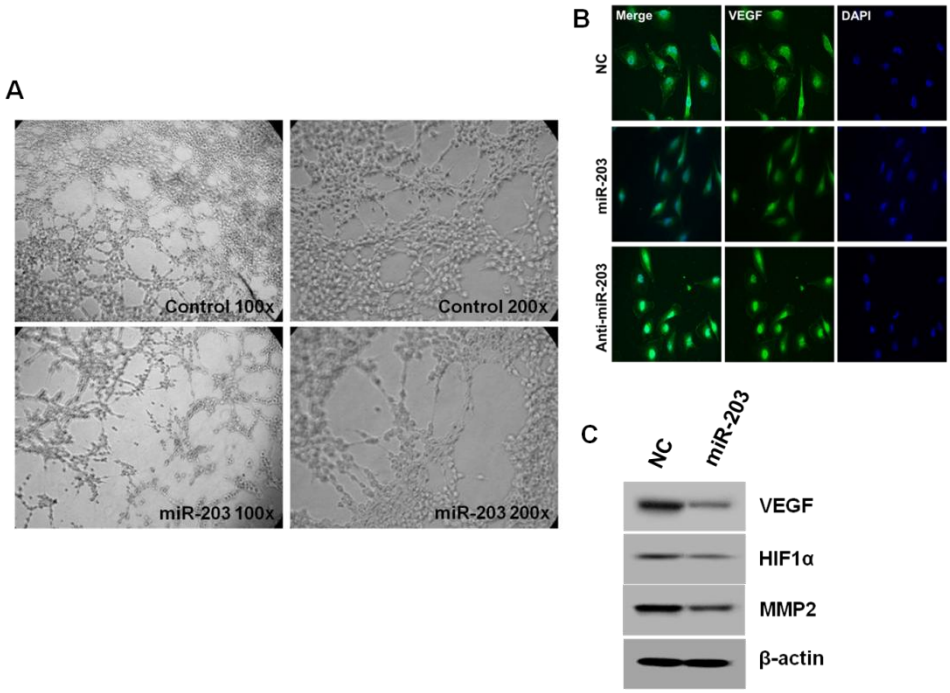

Figure 6. (A) Photographs of representative VM formation fields (B) Immunofluorescence staining for VEGF (C) The western blot analysis (VEGF, MMP2, and HIF1-α). The effect of miR-203 on VM formation in the U251 cells was determined. (A) Photographs of representative VM formation fields are shown. The upper panel shows the control group transfected with negative-control pre-miRNA, and the lower panel shows miR-203-transfected U251 cells (left panel: 100x magnification, and right panel: 200x magnification). Vasculogenic mimicry formation was significantly reduced in the miR-203-transfected cells. (B) Immunofluorescence staining for VEGF showed decreased VEGF expression in the miR-203-transfected cells compared to the negative control. (C) The western blot analysis showed that the expression of VEGF, MMP2, and HIF1-α decreased with the overexpression of miR-203.

Discussion

To our knowledge, this study is the first to investigate the role of miR-203 in the radiosensitivity of MG cells and its related pathways. These results indicate that the therapeutic index can be enhanced with miR-203 based on the selective radiosensitizing effect on MG cells. Furthermore, we found that multiple pathways and mechanisms are involved in miR-203-related radiosensitivity in MG cells.

We used multiple target prediction algorithms to select the target genes. Although existing algorithms have limitations because full complementarity is not required and the connection is highly complex in miRNA-mRNA interactions in animals (12), the predicted genes from multiple databases helped determine the experiment design of this study and were highly consistent with the western blotting findings.

Because one of the major mechanisms of therapeutic resistance, including of RT, is DNA damage repair, we speculated that miR-203 would impede HR by targeting ATM, from the results of the bioinformatics analysis and from the previous study that confirmed that ATM is a direct target of miR-203 (13). As expected, miR-203 overexpression sustained the radiation-induced DNA damage foci formation, and the defective DNA repair was attributed to the inhibited HR, from the results of the western blotting that showed decreased expression levels of the *p*-ATM and RAD51 genes in the miR-203-transfected cells.

To elucidate the mechanism of miR-203 inhibition of survival signaling pathways, we investigated the key genes thought to be involved in GBM radioresistance, including the PI3K/AKT and JAK/STAT3 signaling pathways.

Those are extensively studied pathways that are involved in radiation resistance by regulating radiation-induced apoptosis and cellular proliferation, due to which they are considered attractive targets for radioresistance in various cancer types, including GBM (14,15). The PI3K/AKT pathway is hyperactivated in GBM, and the PI3K or AKT inhibition showed impairment of double-strand break repair and resulted in radiation sensitivity (16). We previously reported that PI3K/AKT is an important signaling complex in the modulation of the radiation response in human cancer cells (10,17). Additionally, we found that miR-203 repressed PLD2. PLD2 is thought to be related to the PI3K/AKT pathway by directly activating AKT, and is considered a novel target for GBM because the PLD2 inhibition showed decreased invasion and migration (18). Our result is consistent with that of the study of Chen et al., which showed that miR-203 inhibits the growth potential of MG cells by directly targeting PLD2 (8). Moreover, we assume that PLD2 will also modulate the radiosensitivity of the MG cells by affecting the PI3K/AKT pathway. STAT3 expression is known to be correlated with the tumor aggressiveness acquired by mesenchymal differentiation and associated with a poor clinical outcome of malignant glioma (19). Importantly, downregulation of STAT3 through modulation of RNAi or SOCS3, the endogenous inhibitor of STAT3, was found to have been correlated to the increased radiosensitivity in the previous studies (20-22). Strikingly, the data presented in this paper show that miR-203 downregulated multiple pathways and that those might altogether contribute to radiosensitization in MG cells.

We observed that miR-203 induced a potential reduction in invasion/migration, the reversal of the EMT-like phenotype, and a decrease in VM.

EMT induces the acquisition of migratory and disseminating features of cancer cells and is thought to be related to radioresistance. Theys et al. showed that E-cadherin-mediated changes in EMT were related to radiosensitivity (23). Recently, Meng et al. performed a Gene Set Enrichment Analysis of a TCGA cohort with GBM patients. The results showed that EMT-related genes were enriched among radioresistant patients (24). VM is a *de novo* tumor microcirculation system that consists of tumor cells that have gained the pattern of endothelial cells, the vasculogenic network of cells with the pattern of embryonic cells, by altering their tumor markers due to their plasticity. EMT is the process of conversion of epithelial cells into mesenchymal stem cells, and is important for embryonic development. Based on the similarity of the VM process and EMT, Du et al. examined the association between VM and EMT and showed that the hypoxia-mediated EMT induction resulted in VM (25). Thus, although the roles of the genes that regulate EMT and VM need further investigation, the reverse of EMT and the reduction of VM shown in this study might autonomously affect tumor aggressiveness and radiosensitivity.

The post-transcriptional modifications shown in this study can also contribute to the modulation of EMT and VM. They can be linked through STAT3, as it is a mediator of multiple oncogenic signaling cascades. STAT3 is involved in modulating the vascular pathway. Vascular- or hypoxia-related genes, which miR-203 also decreased in this study, are known to contribute to the inflammatory microenvironment and therefore, to the regulation of EMT and VM in several tumor types (25,26). VEGF and HIF-1α are angiogenesis markers, and MMP2 is a tissue remodeling marker. They are all related to tumor hypoxia, invasion,

angiogenesis, and metastasis in GBM (27,28). STAT3 directly regulates VEGF gene overexpression and also results in tumor angiogenesis. Its inhibition reduces SRC-induced VEGF expression in vivo (29). Moreover, in a study by Xu et al., STAT3 was crucial in regulating HIF-1α expression via the PI3K/AKT pathway by directly targeting AKT1 (30).

Considering that the connection of pathways to GBM survival, angiogenesis, proliferation, and invasion is extremely complicated, miRNA, as a therapeutic target, is appealing because a single miRNA can have hundreds of target genes, and therefore, regulate multiple pathways at multiple levels (31). However, most studies on miRNA-mRNA interactions have been performed based on single miRNA to single mRNA settings. Relatively little research has been carried out on the pathway-wise regulation of miRNA. In this study, with a single miR-203, we observed alterations in multiple oncogenic pathways related to increased radiosensitivity of MG cells. However, the action of the mechanism of miRNA and networks of biological signaling pathways of GBM remain unknown. Therefore, further experiments are needed to understand the complex link between miR-203 and biological pathways, and the unraveling of DNA repair networks.

This study demonstrated that miR-203 potentially controls the DNA damage repair pathway via the PI3K/AKT and JAK/STAT3 pathways, and that these may collectively contribute to the modulation of radiosensitivity in MG cells by inhibiting DNA damage repair, pro-survival signaling, and EMT. Taken together, our findings suggest that miR-203 could be a useful target for overcoming the radioresistance of GBM.

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국문초록

마이크로 RNA-203 이 악성뇌교종세포의 방사선감수성에 미치는 영향

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연구목적: 본 연구에서는 miR-203이 교모세포종의 방사선감수성에 미치는 영향 및 그에 관여하는 표적유전자에 대해 연구하고자 하였다.

연구 방법: 세 종류의 악성 뇌교종 세포주와 정상 성상교세포 들을 각각 대조군 마이크로RNA, pre-miR-203, antisense miR-203 에 형질주입하였다. RT-PCR, 집락형성 분석, 면역형광법, 침윤/이동 분석 등을 시행하였고 표적유전자의 예측을 위하여 마이크로RNA 표적 데이터베이스에서의 생물정보학적 분석이 사용되었다.

실험결과: miR-203 의 과발현은 세 가지의 인간 악성 뇌교종 세포주 전부에서 방사선감수성을 증가시켰으며, γ-H2AX foci 형성 역시 연장시켰다. 생물정보학적 분석을 통하여 miR-203 가 DNA 복구의 전사 후 조절, PI3K/AKT, SRC, JAK/STAT3, 혈관관련 신호 전달 경로와 연관되었을 가능성이 높음을 알아내었고, western blot 분석을 통해 miR-

203 이 ATM, RAD51, SRC, PLD2, PI3K-AKT, JAK-STAT3, VEGF, HIF-1α, MMP2 를 억제시킴을 확인하였다. miR-203의 과발현은 침윤/이동능을 감소시키고, SLUG 와 Vimentin 을 downregulation 시킴과 동시에 Claudin-1 과 ZO1 를 upregulation 하였다.

결론: 이상의 결과들을 통해 miR-203 가 PI3K/AKT 및 JAK/STAT3 pathway 를 통해 DNA 손상 복구를 조절하고, 또한 악성 뇌교종세포에서 DNA 손상의 복구, pro-survival signaling, 그리고 상피간엽이행을 억제하여 방사선감수성을 조절할 수 있음을 알아냈다. 따라서, 교모세포종의 방사선저항성을 극복하기 위하여 miR-203 이 새로운 표적이 될 수 있을 것이라고 생각된다.

주요어: 마이크로RNA-203, 교모세포종, 방사선감수성

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