

Analysis of *p53* and miRNA Expression after Irradiation of Glioblastoma Cell Lines

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Abstract. Glioblastoma is a malignant brain tumor that is difficult to completely cure by surgical treatment alone. However, resistance to anticancer drugs and radiation may be acquired during treatment. For instance, miRNAs involved in regulating the expression of genes inducing apoptosis and other specific genes have been proposed for use, in order to induce the apoptosis of radioresistant cancer cells. A172 glioblastoma cells, expressing wild-type *p53* were irradiated to a total dose of up to 60 Gy allowing us to analyze the activities of apoptosis-related proteins. The miR-34a expression levels in cells after irradiation at 30 and 60 Gy were 0.17- and 18.7-times the *BCL2* and *caspase-9* expression levels, respectively. The high miR-34a expression level in the cells after irradiation at 60 Gy reduced the *p53* expression level. This study suggests that apoptosis might be promoted by regulating the action of miRNAs, even in cells that have acquired radioresistance.

Glioblastoma is an extremely malignant primary tumor of the brain stem associated with a particularly poor prognosis. Patients with this tumor are generally treated effectively using a combination of surgical resection, chemotherapy, and radiotherapy. The indications for radiotherapy are residual tumor and scattered lesions. Proliferating and oxidized cells are particularly sensitive to radiotherapy and are, thus, associated with marked treatment outcomes (1). While radiosensitivity differs among tumors, the high proliferative ability of glioblastoma makes it highly sensitive to radiation,

and its growth is temporarily halted after radiotherapy; however, the acquisition of radioresistance during treatment may result in local recurrence.

In recent years, molecularly-targeted therapy targeting specific genes has attracted attention as a replacement for conventional treatment methods, such as anticancer drug therapy. Micro-RNAs (miRNAs) are also targets of molecularly-targeted therapy, and those involved in apoptosis or regulating the expression of specific genes could potentially induce the apoptosis of cancer cells exhibiting radioresistance (2).

We propose that the identification of miRNAs that inhibit the expression of *p53* gene, which regulates DNA damage-induced apoptosis, could enable the induction of apoptosis of radioresistant cells or cells with a *p53* gene mutation. In this study, A172 glioblastoma cells expressing wild-type *p53* were irradiated to a total dose of 60 Gy to produce radioresistant cell strains. We then analyzed the activities of apoptosis-related proteins *p53*, *BCL2*, *BAX*, *caspase-9*, and *caspase-3* in these cells in the presence and absence of miRNAs that regulate apoptosis.

Materials and Methods

Cell culture. In A172 human glioblastoma cells (Japanese Cancer Resources Bank, Osaka, Japan), we added 10% inactivated fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin GIBCO Penicillin-Streptomycin liquid (Invitrogen, CA, USA) in Dulbecco's Modified Eagle's Medium/Ham's F-12 culture medium. Cells were incubated at 37°C with 95% air/5% CO₂. We used the human GBM cell line A172 derived from the Japanese Cancer Resources Bank. The cells were irradiated with 2 Gy once a day. Cells were irradiated until the total 60 Gy. Then, irradiated (30 Gy and 60 Gy) and non-irradiated (control) A172 cells were cultured.

Irradiation conditions. Irradiation parameters were set as follows: quality, 4 MV/X-rays; dose rate, 2 Gy/min; field, 25×25 cm; irradiation depth, 4.0 cm using a MEVATRON KD2/50 (TOSHIBA, Tokyo, Japan). Cells were cultured at 2×10⁵ cells/dish in 10-cm

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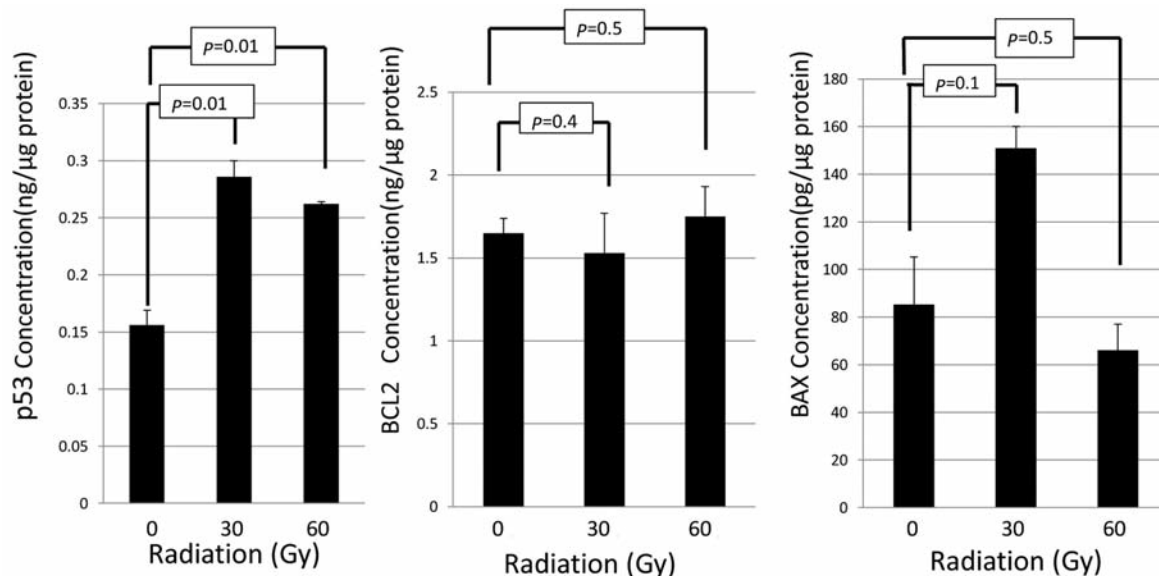


Figure 1. Analysis of Enzyme-Linked ImmunoSorbent Assay (ELISA) measurement of p53 and BAX and BCL2. The p53 expression level was significantly higher in the cells, after 30 Gy irradiation, than in the control cells. There was no difference in the BAX and BCL2 expression level between 30 and 60 Gy of irradiation.

dishes for irradiation. The cells were irradiated with 2 Gy once a day. The cells irradiated to 60 Gy were used for protein and miRNA analysis.

Enzyme-Linked ImmunoSorbent Assay (ELISA) for apoptosis factors. We centrifuged 1×10^6 irradiated and non-irradiated control cells in 1.5-ml tubes, washed the pellet in phosphate-buffered saline, and then added Cell Lysis Buffer 4 (80-1339; Enzo Life Sciences, NY, USA), containing 1 mM Phenylmethylsulfonyl fluoride and a Protease Inhibitor Cocktail (Sigma P8340, 0.5 μl/ml, St. Louis, USA). The sample was cooled on ice for 15 min and then re-centrifuged. Supernatant samples were used to measure p53 with a TP53 ELISA (E90928Hu; Uscn Life Science, Wuhan, China) and BCL2 with the BCL2 ELISA kit (E90778Hu; Uscn Life Science) in a leader fluorospectrophotometer plate ($\lambda=450$ nm). Then 4×10^5 cells/dish were washed in phosphate-buffered saline and mixed with Cell Lysis Buffer 4, as above. BAX levels were measured using the ELISA kit for human BAX (ADI-900-138; Enzo Life Sciences) in a leader fluorospectrophotometer plate (λ excitation=450 nm, emission=590 nm).

Caspase activity assay. Caspase-9 activity was measured using the substrate LEHD (Leu-Glu-His-Asp)-7-amino-4-trifluoro methyl coumarin (AFC) (cat.#218748; Calbiochem, Darmstadt, Germany), while the caspase-3 substrate was Ac (N-acetyl) -DEVD-AFC (7-amino-4-trifluoromethylcoumarin) -DEVD-7-amino-4-trifluoromethylcoumarin (AFC), Kamiya Biomedical, USA). A total of 1×10^6 cells/dish were washed in phosphate-buffered saline as before and Cell Lysis Buffer 4 was added on ice for 10 min. The cell lysates were then mixed with the reaction buffer 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.5 with 20% glycerol, 5 mM DTT, 0.5 mM EDTA, and assayed in a leader fluorospectro photometer plate (λ excitation=400 nm, emission=505 nm).

miRNA measurement. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). We reverse-transcribed miRNAs after extraction using a RT² qPCR-Grade miRNA Isolation Kit (MA-1; SABiosciences, Tokyo, Japan) from 10-40 μg total RNA to form cDNA. An ABI PRISM 7000 Sequence System (Applied Biosystems, Inc. California, USA) was then used under the following conditions: 95°C for 10 min (1 cycle), PCR denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec (PCR conducted in 40 cycles). Data were analyzed by the $\Delta\Delta$ Ct method (original expression level= $2^{-\Delta\Delta Ct}$). Data are expressed at the Ct level of the cells subjected to irradiation as a ratio of that of non-irradiated the cells.

Statistical analysis. Data were tested for statistical significance using ANOVA. Results are expressed with statistical significance set at $p < 0.05$.

Results

Analysis of ELISA measurements. The p53 expression level was 0.156 ng/μg of protein in the control cells, 0.286 ng/μg ($p=0.01$) after 30 Gy irradiation, and 0.262 ng/μg ($p=0.01$) after 60 Gy irradiation. The Bcl-2 expression was 1.65 ng/μg of protein in the control cells, 1.53 ng/μg ($p=0.6$) after 30 Gy irradiation, and 1.75 ng/μg ($p=0.5$) after 60 Gy irradiation. The Bax expression level determined using ELISA was 85.3 pg/μg of protein in the control cells, 151 pg/μg ($p=0.04$) after 30 Gy irradiation, and 66 pg/μg ($p=0.04$) after 60 Gy irradiation (Figure 1).

The caspase-9 activity was 25 nmol/mg protein/h in the control cells, 2,316 nmol/mg protein/h after 30 Gy irradiation, showing a significant increase, and 1,118 nmol/mg protein/h

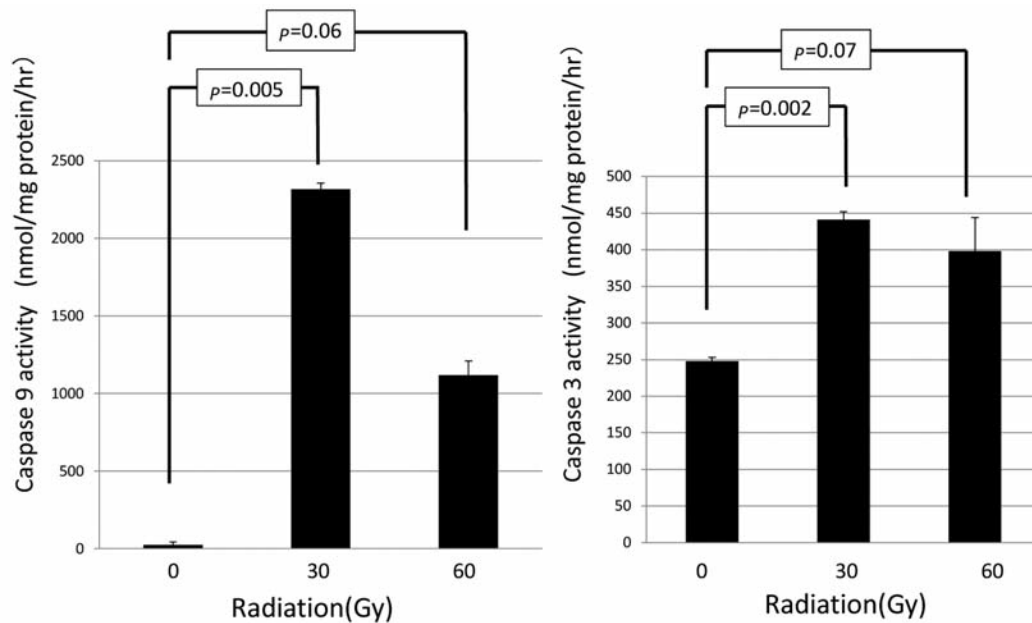


Figure 2. Caspase-3 and caspase-9 activity was significantly higher in the 30 Gy-irradiated cells than in the control cells, suggesting a decrease in the apoptosis induction compared with the cells after 30 Gy irradiation. There was no difference in the expression level between control and 60 Gy of irradiation. Decreases in caspase activities indicate the acquisition of radioresistance with an increase in the radiation dose.

after 60 Gy irradiation, also showing an increase ($p=0.065$). Caspase-3 activity was 248 nmol/mg protein/h in the control cells, 441 nmol/mg protein/h ($p=0.001$) after 30 Gy irradiation, and 398 nmol/mg protein/h ($p=0.07$) after 60 Gy irradiation (Figure 2).

Analysis of miRNA expression. The miR-34a expression levels in cells after irradiation at 30 and 60 Gy were 0.17- and 18.7-times that of the control cells, respectively. The miR-21 expression level in cells after 30 Gy irradiation was 4.08-times that of the control, but no miR-21 expression was observed after 60 Gy irradiation. The miR-222 expression level in cells after 30 Gy irradiation was 1.29-times that in the control cells, but no miR-222 expression was observed after 60 Gy irradiation (Figure 3).

Discussion

This study found that both p53 and BAX expression levels were significantly higher in glioblastoma cells subjected to irradiation at 30 Gy, compared to control, non-irradiated cells. The cells also exhibited significantly higher caspase-9 and caspase-3 activities after 30 Gy irradiation than did the control cells, indicating a high apoptosis induction activity after irradiation at the specific dose. Apoptosis is induced more readily by irradiation in proliferating cells because radiosensitivity depends on the cell cycle at the time of irradiation, increasing in the latter half of the S phase,

immediately before DNA synthesis, and decreasing to radioresistance after the initiation of DNA synthesis (3). After irradiation, the high-order function of DNA changes, and irradiation from the G to S phase causes chromosomal pattern abnormalities, while irradiation in the S to G₂ stage results in abnormalities of the chromatid pattern. Since cells with pre-existing chromosomal abnormalities undergo cell-cycle arrest at the cell-cycle checkpoints, leading to apoptosis, cells after 30 Gy irradiation cannot repair chromosomes, and thus frequently take the path to apoptosis (4).

After 60 Gy irradiation, glioblastoma cells showed a significantly higher p53 expression level, a significantly lower Bax expression level, a significantly higher caspase-9 activity, and a slightly higher caspase-3 activity than the control cells, suggesting a decrease in apoptosis induction compared with the cells after 30 Gy irradiation. Decreases in BAX and caspase activities indicate the acquisition of radioresistance following an increase in the radiation dose, and then radioresistance hinders apoptosis (Figure 4).

These results suggest that cell death reached a peak at 30 Gy, at which point high-level radiosensitivity was achieved. Thereafter the radiosensitivity gradually decreased, resulting in a decrease in the apoptosis rate. In other words, radioresistance was acquired at a dose of more than 30 Gy, resulting in a decreased ability of cells to undergo apoptosis.

The ability of miRNAs to inhibit protein synthesis by binding to the 3'UTR of mRNA suggests a potential role in gene and protein regulation (5, 6). To this end, miR-34a has been

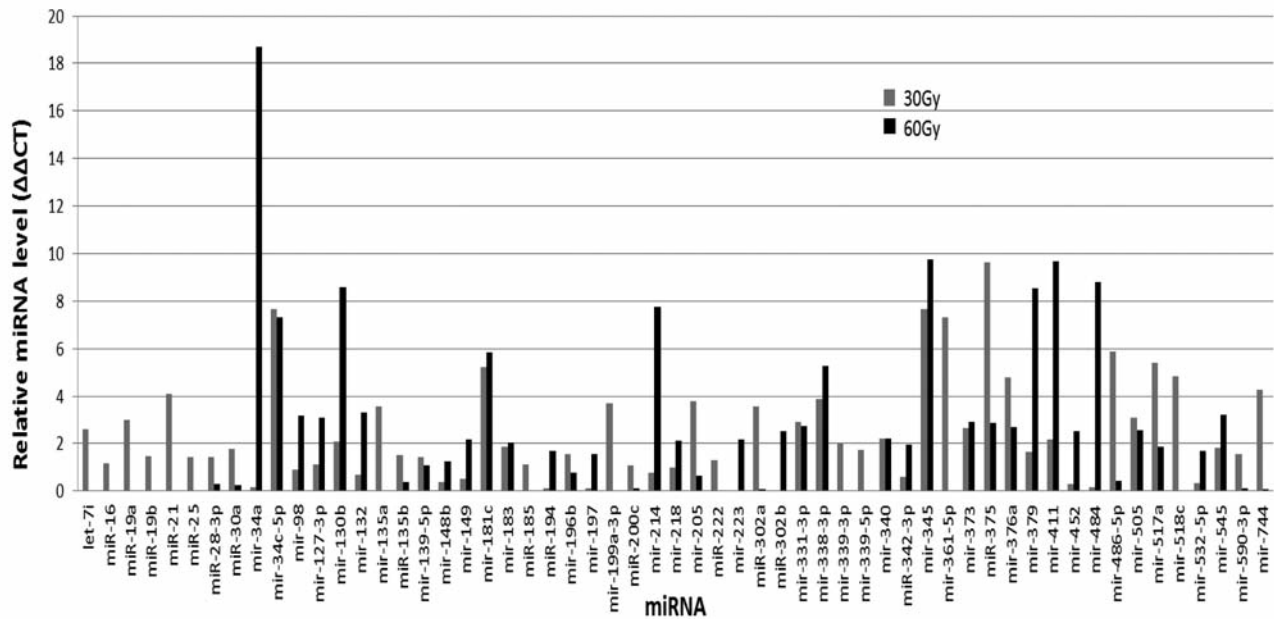


Figure 3. Radiation cells $\Delta\Delta C_t$ / non-radiation cells $\Delta\Delta C_t$. The 30-Gy postradiation cells of A172 cells and the microRNA expression of 60-Gy post-radiation cells. The microRNA manifestation analysis that emerged only in 30Gy is *Let7i*, *miR-16*, *-19a*, *-19b*, *-21*, *-25*, *-135a*, *-185*, *-199a-3p*, *-222*, *-339-3p*, *-339-5p*, *-518c*. The microRNA manifestation analysis that emerged only in 60 Gy is *miR-223*. The microRNA analysis that fell in to in 60 Gy in 30 Gy highly is *miR-28-3p*, *-301*, *-34C-5p*, *-35b*, *-139-5p*, *-196b*, *-200c*, *-205*, *-302a*, *-331-3p*, *-375*, *-376a*, *-486-5p*, *-505*, *-517a*, *-590-3p*, *-744*. The microRNA analysis that highly in 60 Gy in 30 Gy low is *miR-34a*, *-98*, *-127-3p*, *-130v*, *-132*, *-148b*, *-149*, *-181c*, *-183*, *-194*, *-197*, *-214*, *-218*, *-338-3p*, *-342-3p*, *-345*, *-373*, *-379*, *-411*, *-452*, *-484*, *-532-5p*, *-545*.

evaluated as a target factor regulating p53 in chemotherapy regimens for medulloblastoma (7). In the present study, *miR-34a* expression levels in the glioblastoma cells after 30 Gy and 60 Gy irradiation were 0.17- and 18.7-times, respectively, higher than the level in control cells. Since the protein expression level is low when the miRNA expression level is high, the inhibition of p53 expression in the cells irradiated at 60 Gy compared with those after 30 Gy irradiation may have been due to the higher *miR-34a* expression level, as well as the acquisition of radioresistance. In the cells after 30 Gy irradiation, the low *miR-34* expression level may have increased the p53 expression level, which promotes downstream gene transcription, increasing apoptosis induction. The *miR-34a* expression level in the cells after 60 Gy irradiation was 18.7 times that in the control cells and 110-times that in the cells after 30 Gy irradiation. In contrast, the high *miR-34a* expression level in the cells after 60 Gy irradiation reduced the p53 expression level, inhibiting downstream gene transcription and apoptosis. miRNA levels have been linked to defective DNA repair pathways and radiosensitivity, which impacts on the regulation of cell death and proliferation after irradiation (8-10). Our results suggest that regulating *miR-34a* expression might induce apoptosis even in glioblastoma cells that have acquired radioresistance. BAX regulation has been reported for *miR-21* (11, 12) and *miR-222* (13). Here, the *miR-222* expression level in the cells after 30 Gy irradiation was 1.29-times that in the control cells, while the

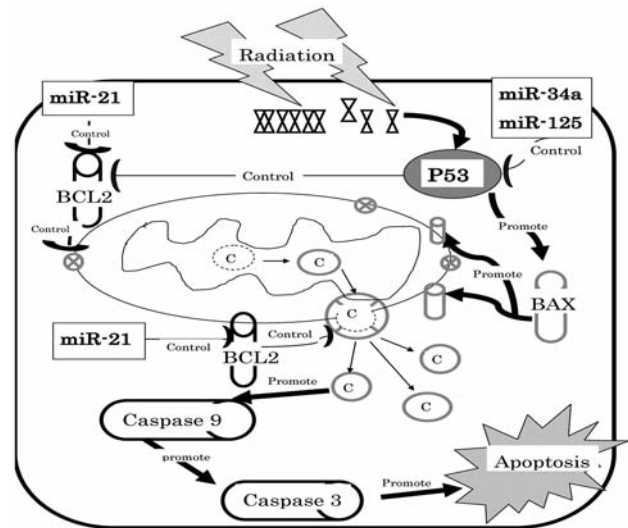


Figure 4. Apoptosis pathway following irradiation. miRNAs inhibit protein synthesis by binding to the 3' UTR of mRNA and by altering gene regulation. Accordingly, applications of miRNAs have been reported including *miR-34a* as a target factor regulating p53 and *miR-21*/*miR-222* for regulating BAX (9).

miR-21 expression level in the cells, after 30 Gy irradiation, was 4.08-times that in the control cells, but no *miR-21* expression was observed after 60 Gy irradiation. In the cells after 30 Gy

irradiation, although both the miR-21 and miR-22 expression levels increased compared with the control cells, the miR-222 level also increased 1.29-fold compared with the control cells, suggesting that the BAX expression level in irradiated cells is not regulated by miR-21 or miR-222. In glioblastoma cells, both BCL2 and BAX expressions are regulated by miR-21. In our cells after 30 Gy irradiation, the miR-21 expression level was 4.08-times that in the control cells, while the BCL2 expression was 0.92-times that in the control cells, whereas cells irradiated at 60 Gy irradiation exhibited no miR-21 expression. However, since there was no difference in the BCL2 expression level between 30 and 60 Gy of irradiation, BCL2 expression in irradiated cells may not be regulated by miR-21.

Irradiation-activated p53 induces apoptosis by binding to the transcriptional regulatory region of transcriptional target genes. Therefore, p53 is being evaluated as a possible target of chemotherapy and radiotherapy treatments for glioblastoma (14-16). The p53 pathway plays an important role in the inhibition of cancer cells. However, due to radiation-induced DNA damage, the p53 DNA-binding protein TP53BP1 promotes p53 accumulation leading to radioresistance, and its gene expression level increases with the radiation dose. As a result, radioresistance increases, inhibiting the transcriptional activity of genes inducing apoptosis, and treatment effects decrease (14). This study suggests that apoptosis could be promoted by regulating the action of miRNAs, even in cells that have acquired radioresistance.

Conclusion

This study found that both p53 and BAX expression levels were significantly higher in glioblastoma cells subjected to irradiation at 30 Gy of irradiation than in control, non-irradiated cells. Results suggest that regulating miR-34a expression might induce apoptosis even in glioblastoma cells that have acquired radioresistance. This study suggests that apoptosis could be promoted by regulating the action of miRNAs, even in cells that have acquired radioresistance.

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