Abstract. Radioresistance remains a major challenge in the treatment of glioma, and the response of patients to radiotherapy varies considerably. MicroRNAs (miRNAs) are involved in various biological processes. The purpose of the present study was to investigate miRNAs involved in the response to radiation in glioma cell lines. Total RNA was isolated from human glioma U251 cells 30 min after γ-ray exposure and hybridized to an miRNA chip array. miRNA expression profiles were analyzed by quantitative real-time PCR. pcDNA3/EGFP-miR-212 mimic transfection was used to verify the function of miR-212 in colony formation tests, and the effect of miR-212 overexpression on U251 cells was examined by western blot analysis of apoptosis-related proteins (Bcl-2, Bax, caspase-3 and cytochrome c). The target genes of miR-212 were predicted using bioinformatic tools including miRNA databases, and breast cancer susceptibility gene 1 (BRCA1) was selected for further confirmation by EGFP fluorescence reporter and loss- and gain-of-function assays. Of the 16 candidate miRNAs showing altered expression, five were assessed by real-time PCR; miR-212 was identified as contributing to the radioresistance of glioma cells and was shown to attenuate radiation-induced apoptosis. miR-212 negatively regulated BRCA1 expression by interacting with its 3'-untranslated region, suggesting a correlation between BRCA1 expression and radiosensitivity in glioma cells. U-118MG and SHG-44 cell lines were used to confirm these observations. The response of glioma cells to radiation involves the miR-212-mediated modulation of BRCA1 gene expression, suggesting that the miR-212/BRCA1 axis may play a potential role in the radiotherapy of gliomas.

Introduction

Breast cancer susceptibility gene 1 (BRCA1) is a tissue-specific tumor suppressor involved in basic cellular functions necessary for cell replication and DNA synthesis (1-3). A large body of evidence (4-6) indicates that BRCA1 is involved in several important cellular pathways, including DNA damage repair, chromatin remodeling, and checkpoint activation. BRCA1 affects the outcomes of chemotherapy or radiotherapy for the treatment of breast cancer (7), although whether it increases or decreases the chemo/radiosensitivity of different cancers remains unclear.

Malignant gliomas, including multitype and mixed gliomas, are the most common and deadly malignant primary brain tumors and are associated with poor prognosis and rapid invasion. The median survival of patients with the most aggressive type of malignant glioma is 12-14 months, despite the availability of treatments such as surgery, chemotheraphy and/or radiotherapy (8,9). Therefore, new strategies to enhance the therapeutic effect of chemo/radiotherapy are urgently needed.

MicroRNAs (miRNAs) are small-non-coding RNAs that regulate gene expression at the post-transcriptional level through complete or incomplete complementary binding to the 3'-untranslated region (3'-UTR) of target genes (10). miRNAs play an important role in the regulation of gene expression, and they mostly function as negative regulators (11), with involvement in positive gene regulation in fewer cases (12,13). miRNAs regulate approximately 60% of protein-coding genes and participate in several biological processes at multiple steps. They play an important role in tumor development by affecting tumor cell growth, differentiation, and apoptosis, and the cell cycle. miRNAs can function as tumor suppressors or oncogenes in cancer depending on the genes or pathways that they regulate. Growing evidence (9,14,15) indicates that miRNAs affect the therapeutic response to chemotheraphy or radiotherapy, which opens new avenues for the diagnosis of cancer.
and prognosis of tumors and identifies potential therapeutic targets to improve patient survival. miR-212, which is located at chromosome 17p13.3, is overexpressed in many cancers, including non-small cell lung cancer (16) and oral carcinoma (17), whereas it is downregulated in other tumors such as hepatocellular carcinoma (18), gastric cancer (19,20), colorectal cancer (21), and prostate cancer (22). In the present study, we explored the role of miR-212 in the radiosensitivity of the U251 human glioma tumor cell line. miR-212 was identified as a radiation-induced miRNA by gene chip screening and confirmed by fluorescence quantitative PCR. We identified BRCA1 as a target of miR-212, and examined the role of the association between BRCA1 and miR-212 in the radiosensitivity of glioma cells. Gain- and loss-of-function assays showed that miR-212 contributes to the radioresistance of glioma cells. To the best of our knowledge, this is the first study reporting the role of miR-212 in the radiosensitivity of glioma tumor cells and the possible underlying mechanism. Our findings may help to develop a novel miRNA-based therapeutic strategy which combines miR-212/BRCA1 interaction with radiotherapy for glioma.

Materials and methods

Cell lines and γ-irradiation. Human glioma U251, U-118MG and SHG-44 cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and cultured in DMEM supplemented with 10% dialyzed fetal bovine serum (FBS) and 1% PS (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C. The cells were transfected with pcDNA3/hsa-miR-212 or the pcDNA3 control, antisense oligonucleotide (ASO)-miR-212 or ASO-ctrl, pcDNA3/EGFP, pcDNA3/EGFP-BRCA1, or pcDNA3/EGFP-BRCA1 3'-UTR mut using Invitrogen Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Cells were exposed to average doses of 157Cesium-γ-irradiation at a rate of 0.8 Gy/min.

RNA isolation, miRNA chip array, and real-time quantitative RT-PCR assay. RNA isolation and miRNA chip array were performed at 30 min after γ-ray exposure. Total RNA was isolated with TRIzol (cat. no. 15596-026, Invitrogen; Thermo Fisher Scientific) and pretreated with a wash buffer and 100 µg/ml streptomycin). Cells were maintained in a humidified incubator with 5% CO₂ at 37°C. The cells were transfected with pcDNA3/hsa-miR-212 or the pcDNA3 control, antisense oligonucleotide (ASO)-miR-212 or ASO-ctrl, pcDNA3/EGFP, pcDNA3/EGFP-BRCA1, or pcDNA3/EGFP-BRCA1 3'-UTR mut using Invitrogen Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The miRCUR™ Array (Exiqon) was performed with a GenePix 4000B scanner and the GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA) using the following parameters: wavelength, 532 nm, Cy3; 532 PMT gain, 650 (default, modified as indicated); power, 33%; pixel size, 10 µm; lines to average, 1; and focus position, 0 µm. Specific primers were used for the miRNA reverse transcription (RT) reaction, whereas the cDNA of genes was reverse transcribed using oligo(dT) primers. The PCR assay was performed using the SYBR Green PCR Master Mix (cat. no. 4367659, ABI) and analyzed using the ABI Prism 7900 (both from Thermo Fisher Scientific). The reaction conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 15 sec, 65°C for 15 sec, and 72°C for 32 sec. Melting curve analysis was performed at 60-95°C. The primers for RT and PCR are shown in Table I. The U6 small nuclear B non-coding RNA (RNU6B) was used as the endogenous control to normalize the level of miR-212.

Plasmid construction. The primary miR-212 sequence was amplified from genomic DNA and cloned into the pcDNA3/EGFP vector at BamHI and EcoRI sites. The gene coding BRCA1 was amplified from the cDNA of MCF-7 cells and cloned into the pcDNA3 vector at NheI and ApaI sites. The shRNA of BRCA1 was annealed and cloned into the pcDNA3 vector at BamHI and HindIII sites. The 3'-UTR of BRCA1 (containing the binding sites for miR-212) was amplified from cDNA of MCF-7 cells. The product was cloned into the pcDNA3-EGFP control vector (downstream of EGFP). The mutant 3'-UTR of BRCA1 (four nucleotides were mutated in the binding site) was amplified from the construct pcDNA3-EGFP/BRCA1 3'-UTR. The primers used for PCR amplification are listed in Table I.

EGFP fluorescent reporter assay. U251 cells were co-transfected with pcDNA3/miR-212 or ASO miR-212 and the 3'-UTR of BRCA1 or the mutant 3'-UTR of BRCA1 or with the control vectors in 48-well plates. At 48 h after transfection, the fluorescence intensity was measured with the F-4500 fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The vector pDsRed2-N1 (Clontech Laboratories, Inc., Mountainview, CA, USA) expressing RFP was transfected together with the above vectors and used as the spiked-in control.

Western blot analysis. Cells were washed with PBS and lysed on ice in RIPA buffer (V900854, Sigma) containing a cocktail of protease inhibitors (P8340, Sigma) following the manufacturer's protocols. Protein concentration was measured using the BCA assay. Protein fractions were suspended in loading buffer and denatured at 100°C for 5 min. Total proteins (20 µg/lane) were separated on 12% SDS-PAGE and transferred to PVDF membranes, which were blocked in 5% fat free milk in TBST buffer (0.1% Tween-20) for 2 h at room temperature. BRCA1 levels were analyzed using a mouse monoclonal anti-BRCA1 antibody (1:1,000; ab16780; Abcam); Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, and cytochrome c levels were detected using the following mouse or rabbit monoclonal antibodies at a dilution of 1:1,000: ab117115, ab5714, ab13586, ab32042, and ab13575, respectively (Abcam). The secondary antibodies used were goat anti-rabbit antibody (1:1,000; Sc2030; Santa Cruz Biotechnology) and rabbit anti-mouse antibody (1:1,000; Sc538917; Santa Cruz Biotechnology). GAPDH was used as the endogenous control to normalize the expression levels of the proteins of interest. GAPDH levels were detected using HRP-conjugated mouse anti-GAPDH monoclonal antibody (1:5,000; HRP-60004, Proteintech). The densitometry scan of the western blotting was performed by Image Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Colonies formation assay. Cells were seeded into 12-well plates at a density of 1000 cells/well at 24 h after transfection. The medium was changed every 3 days. When most of the colonies contained at least 50 cells, they were fixed in 100% methanol.
and stained with crystal violet and counted. The colony formation rate was calculated as (colony number)/(seeded cell number).

**TdT-mediated dUTP nick-end labeling (TUNEL) assay.** Cells irradiated at doses of 0, 5, and 10 Gy were seeded into 14-well plates at a density of 2000 cells/well at 48 h after transfection. After 24 h, the cells were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized in 0.5% Triton X-100. Then, the cells were washed with PBS three times, followed by a 1-h incubation at 37˚C with Terminal deoxynucleotidyl Transferase (TdT), and the cells were stained with fluorescein isothiocyanate-dUTP. Finally, the cells were stained with DAPI for 5 min at room temperature. The slides were examined with a fluorescence microscope.

**Statistical analysis.** Each experiment was repeated at least twice, and data are shown as the mean ± SD. The statistical analysis between two groups was performed by two-tailed Student’s t-test. For the statistical analysis of the rescue experiment with four groups, a one-way analysis of variance test and the least significant differences t-test were performed. A value of P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-212 is downregulated in human glioma U251 cells following irradiation.** The miRNA expression profile of U251 cells exposed to γ-radiation (at doses of 5 and 10 Gy) was analyzed by chip array assay. The signal intensity of miRNAs relative to that of unirradiated U251 cells as controls is shown in Fig. 1A. Of the 16 miRNAs showing alterations in expression, five were further examined by real-time PCR, that is, miR-212, miR-96, miR-18a, miR-21, and miR-423 (Fig. 1B). The relative expression levels of these five miRNAs relative to that of U6 as the endogenous control are shown in Fig. 1C. Of the five miRNAs analyzed, miR-212 was identified as a negatively associated radiation-induced miRNA that was downregulated in human glioma cells after radiation exposure. This was confirmed in SHG-44 glioma cells by real-time PCR (data not shown).

**miR-212 promotes cell colony formation in U251, U-118MG and SHG-44 cell lines exposed to γ-radiation.** To determine whether miR-212 affects the radiosensitivity of glioma cells, a colony formation assay was performed in the U251, U-118MG (data not shown) and SHG-44 (data not shown) cell lines. miR-212 was overexpressed by transfecting cells with pcDNA3/EGFP-miR-212 mimics, which resulted in a 9-fold higher expression level of miR-212 than that in cells transfected with the pcDNA3/EGFP-mimics control (Fig. 2A). As shown in Fig. 2B, overexpression of miR-212 increased the colony formation rate of U251 cells by ~2-fold compared with that in the controls in the groups receiving 5, and 10 Gy radiation; but in groups without radiation exposure, miR-212 transfection did not significantly affect colony formation. These data indicated that miR-212 increased colony formation ability in response to radiation, suggesting that it induces radioresistance. In addition, the colony number did somewhat decrease in response to an increasing radiation dose despite miR-212 overexpression, indicating that miR-212 did not fully abrogate the effect of radiation.

**miR-212 attenuates radiation-induced apoptosis and affects the expression levels of apoptosis-related proteins.**

| Primers                      | Sequences                                      |
|------------------------------|------------------------------------------------|
| hsa-miR-212-5p RT            | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| hsa-miR-212-5p forward       | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| hsa-miR-96-5p RT             | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| hsa-miR-96-5p forward        | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| hsa-miR-21-3p RT             | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| hsa-miR-21-3p forward        | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| hsa-miR-423-5p RT            | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| hsa-miR-423-5p forward       | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| U6 RT                        | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| U6 reverse                   | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| BRCA1 sense                  | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| BRCA1 antisense              | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| BRCA1 mut sense              | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| BRCA1 mut antisense          | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
| β-actin sense                | 5'-GTCGTATCCAGTCAGGGTCCAGGTGCACCTGGGATACGACAGTAAGCA-3' |
| β-actin antisense            | 5'-TGCGGAGCTTTGGCTCTAGACT-3'                   |
Apoptosis is a common response to irradiation in most cells. In the present study, the potential role of miR-212 in radiation-induced apoptosis was tested by fluorescence microscopy assay and confirmed by western blot analysis of apoptosis-related proteins such as Bcl-2, Bax, caspase-3, and cytochrome c, with GAPDH as the endogenous control. As shown in Fig. 3A, overexpression of miR-212 (miR-212 mimics) attenuated radiation-induced apoptosis compared...
with that in cells transfected with the mimic control, whereas overexpression of miR-212 had no effect on unirradiated U251 cells. Consistent with this result, western blot analysis indicated that miR-212 overexpression attenuated the radiation-induced down regulation of Bcl-2 and upregulation of cleaved-caspase-3, but there was no significance change in Bax or cytochrome c (Fig. 3B). These results suggest that miR-212 plays a role in radiation-induced apoptosis.

**miR-212 directly targets BRCA1 and negatively regulates its expression.** To explore the mechanism underlying the inhibition of radiation-induced apoptosis by miR-212 in human glioma cells, three algorithms were used to predict the target genes of miR-212, namely, microRNA, TargetScan, and PicTar. Among the predicted genes, we selected BRCA1 as our candidate target. The binding sites for miR-212 on the 3'-UTR of BRCA1 are shown in Fig. 4A. The results of the EGFP fluorescence reporter assay (Fig. 4B) showed that endogenous miR-212 suppressed the upregulation of BRCA1 induced by pcDNA3/EGFP-BRCA1 transfection by ~55% (Fig. 4C) compared with that in cells transfected with pcDNA3/EGFP. Co-transfection with pcDNA3/EGFP-BRCA1 and pcDNA3/EGFP-hsa-miR-212 ASO partly reversed this effect, mainly because the pcDNA3/EGFP-hsa-miR-212 ASO eliminated the endogenous miR-212 expression. The fluorescence intensity was restored to ~90% of the levels in cells transfected with pcDNA3/EGFP. The mutant BRCA1 containing vector pcDNA3/EGFP-BRCA1 3'-UTR mut transfected alone or co-transfected with pcDNA3/EGFP-hsa-miR-212 had no significant effect on fluorescence intensity compared with that in cells transfected with pcDNA3/EGFP. Taken together, these results indicated that miR-212 interacted with the 3'-UTR of BRCA1.

To confirm the association of hsa-miR-212 with BRCA1, BRCA1 gene and protein expression levels were measured by real-time PCR and western blotting in response to miR-212 upregulation or downregulation in SHG-44 and U251 cells. As shown in Fig. 5, overexpression of miR-212 (miR-212 mimics) downregulated BRCA1 gene and protein expression, whereas inhibition of miR-212 (miR-212 ASO) had the opposite effect. Consistent with the fluorescence microscopy results, these findings confirmed that miR-212 negatively regulated BRCA1 expression by interacting with its 3'-UTR.

**Inhibition of BRCA1 by siRNA phenocopies the roles of miR-212.** We next sought to determine whether the miRNA-mediated downregulation of BRCA1 can be applied as a molecular treatment strategy for patients with glioma, particularly as a strategy to modulate radiosensitivity. Cells were treated with siRNA targeting BRCA1, and colony formation assay and fluorescence microscopy analysis of apoptosis were performed after exposure to different doses of radiation. Knockdown of BRCA1 enhanced the colony forming ability of U251 cells after radiation (Fig. 6A) and attenuated radiation-induced apoptosis (Fig. 6B), indicating that BRCA1 is positively associated with radiosensitivity in glioma cells. U-118MG and SHG-44 cell lines were used to confirm these observations (data not shown).

Overall, these data indicated that miR-212 is involved in the response to radiation and functions in the regulation of BRCA1 expression, which is important for tumorigenesis and response to treatment.
Figure 4. miR-212 directly targets BRCA1 and negatively regulates its expression. (A) BRCA1 3'-UTR is the target site interacting with miR-212 as predicted by microRNA databases. (B) Fluorescence reporter assay showing that BRCA1 is a direct target gene of miR-212. (C) Relative fluorescence intensity of cells transfected with 3'-UTR + miR-212 ASO pcDNA3/EGFP, pcDNA3/EGFP-BRCA1 3'-UTR, pcDNA3/EGFP-BRCA1 3'-UTR + ASO NC, pcDNA3/EGFP-BRCA1 3'-UTR + hsa-miR-212 ASO, pcDNA3/EGFP-BRCA1 3'-UTR mut + ASO NC, and pcDNA3/EGFP-BRCA1 3'-UTR mut + hsa-miR-212 ASO after 48 h of transfection.

Figure 5. Effect of hsa-miR-212 on BRCA1 expression at the gene and protein levels in SHG-44 and U251 cells assessed by real-time PCR (A) and western blotting (B). *P<0.05.
miRNAs have been investigated extensively in recent years and have been found to function as post-transcriptional regulators of many genes involved in cancer initiation, development, metastasis and the response to radiation (23). miRNAs have therefore been regarded as attractive targets in the development of more powerful therapies (24). Since the ability of miRNAs to act as negative gene regulators allows them to modulate signaling pathways that regulate multiple cellular processes, although numerous miRNAs are involved, only a few can be targeted therapeutically. Concerning radiotherapy, miRNAs have the potential to be used as either radiosensitizers or radioprotectors. Previous studies (16,19,21,25) have revealed many functions of miR-212, such as tumor-promoting properties in NSCLC (16) and proliferation-inhibition properties in gastric cancer (19). To the best of our knowledge, the present study indicated for the first time that miR-212 is associated with the response to radiation by targeting BRCA1. Gain-of-function experiments showed that miR-212 overexpression increased colony formation in U251 cells after radiation exposure by approximately 2-fold compared with that in the control groups. The present data indicated that miR-212 attenuated radiation-induced apoptosis and affected the expression of apoptosis-related proteins.

Furthermore, we used bioinformatic tools to predict target genes, and BRCA1 was chosen as a candidate target. The EGFP fluorescence reporter assay, a direct method for target validation, verified that BRCA1 is a direct target gene of miR-212 (Fig. 4B and C). miR-212 was shown to be a negative regulator of BRCA1 expression, as overexpression of miR-212 downregulated BRCA1 expression at the gene (Fig. 5A) and protein levels (Fig. 5B), consistent with previous reports that a large number of miRNAs regulate gene expression negatively.

We showed that BRCA1 downregulation by miR-212 can be replaced by siRNA, as knockdown of BRCA1 attenuated radiation-induced apoptosis. This indicated that BRCA1 may have a positive correlation with radiosensitivity in glioma cells, in contrast to previous reports in ovarian (26) and other tumors (27). However, BRCA1 has been shown to act as a tumor suppressor, and to be involved in the response to radiation and cisplatin. The role of BRCA1 in DNA damage (6,28) and tumor development has been investigated extensively; however, the precise mechanisms underlying the effect of BRCA1 in radiosensitivity or chemosensitivity considering tumor heterogeneity remain elusive. The BRCA1 status should be more carefully considered in the planning of anti-glioma treatment (29-31).

Our findings revealed that the miR-212/BRCA1 axis was involved in gliomas cell radiosensitivity, which indicates that miR-212-mediated modulation of BRCA1 gene expression plays a potential role in glioma radiotherapy. Further experiments are necessary to better define the role of the association between miR-212 and BRCA1 in radioresistance.
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