Reduced PTEN expression and overexpression of miR-17-5p, -19a-3p, -19b-3p, -21-5p, -130b-3p, -221-3p and -222-3p by glioblastoma stem-like cells following irradiation

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Abstract. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor gene that induces cell apoptosis by inhibiting the PI3K/Akt signaling pathway. Glioblastoma (GBM) is a brain tumor that is resistant to irradiation and chemotherapy and, thus, is difficult to cure. GBM stem-like cells (GSCs) have been implicated as a cause of this resistance. microRNA (miRNA/miR) inhibits the expression of proteins. The objective of the present study was to identify miRNAs that target PTEN, which induces apoptosis, in irradiation-resistant GSCs. When the expression of miRNAs was examined in GSCs irradiated at 60 Gy using the human GBM A172 cell line, the expression of PTEN-targeting miR-17-5p, -19a-3p, -19b-3p, -21-5p, -130b-3p, -221-3p and -222-3p was significantly higher in irradiated GSCs than in non-irradiated cells, and the PTEN expression levels, as revealed by immunostaining, were lower in the irradiated GSCs than in the non-irradiated cells. These results suggested that the expression of PTEN was suppressed through the overexpression of PTEN-targeting miRNAs in GSCs following irradiation.

Introduction

Glioblastoma (GBM) is the most malignant central nervous system tumor. GBM is typically treated with a combination of surgery, radiotherapy and chemotherapy; however, the effect of this treatment is not satisfactory with regard to prognosis. GBM is characterized by a strong resistance to postoperative radiotherapy and chemotherapy. The cause of this resistance may be related to GBM stem-like cells (GSCs).

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Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a tumor suppressor that inhibits the PI3K/Akt pathway. PTEN dephosphorylates the inositol ring D3 position of the substrate, PIP_3, and transfers it to the cell membrane, which inhibits the activation of cell proliferation-inducing Akt and induces cell apoptosis (1,2). The PI3K/Akt pathway was previously shown to be abnormally enhanced and inhibited apoptosis in malignant tumors in which PTEN was deleted and mutated, and this promoted cell proliferation and the process of cells becoming malignant and resistant to irradiation and chemotherapy (3-6).

Nuclear division and necrosis have frequently been detected in GBM tissue of patients predicted to have a survival time of shorter than one year, and tumors were found to rapidly infiltrate the surrounding tissue and disseminate to the meninx (7). A previous study reported that microRNAs (miRNAs/miR) were complementarily bound to a specific target nucleotide of the 3'-untranslated region (UTR) of mRNA and inhibited the expression of mRNA and protein (8). Secretory miRNAs (circulating miRNAs) may be applied clinically, as they are embedded in the granular vesicles of exosomes and are then secreted so that they are stable in body fluids, such as blood and cerebrospinal fluid, and analyzable in histopathological preparations.

The presence of GSCs scattered in GBM tissue has recently been identified as a factor involved in the development of resistance to GBM treatments (9-11). GSCs have been detected among cells that are positive for the surface antigen cluster of differentiation (CD)133, as well as side population cells. GSC were previously shown to be resistant to irradiation and chemotherapy, as they are in the G_0 phase of the cell cycle, which leads to the recurrence of tumors (10,12). Molecular targeted therapies that target GCSs and PTEN are now being investigated (13-15); however, the radiation doses investigated in these studies are lower than those used for cancer therapy in a clinical setting.

The present study analyzed miRNAs in GSCs irradiated at a dose (total 60 Gy) applied in clinical practice to induce apoptosis in GSCs, which are resistant to irradiation and cause recurrence. The objective of this study was to identify miRNAs that targeted PTEN, which induces apoptosis in irradiation-resistant GSCs, using A172 cells.

Materials and methods

Cell line and culture conditions. The human glioblastoma A172 cell line, which was derived from the Japanese Cancer Resources Bank (Japanese Cancer Resources Bank, Osaka, Japan) was used in the present study. The selected culture medium contained 10% fetal bovine serum and 100 mg/ml streptomycin (Gibco penicillin-streptomycin liquid; Gibco Life Technologies, Carlsbad, CA, USA) in Dulbecco's modified Eagle's medium Ham/F12 (Sigma-Aldrich, Oberhaching, Germany). The cells were incubated at 37˚C with 5% carbon dioxide.

Radiation instrument and radiation conditions. The cells were irradiated with 4-MV X-rays at a dose of 2.00 Gy/min over a 25x25-cm field at a radiation depth of 4.0 cm using a Mevatron KD2/50 (Toshiba, Tokyo, Japan) at room temperature. The cells were cultured for 7 h prior to roentgen irradiation. A total of $2x10^5$ cells were irradiated in a 10-cm dish. The radiation conditions used were 2 Gy/day for 5 days over 1 week. The cells were irradiated for six weeks, up to a total dose of 60 Gy.

Isolation of $CD133^+$/$CD133^-$ cells. Magnetic cell sorting (MiniMACS™ Separator and Starting Kit; cat. no. 130-090-312; Miltenyi Biotec GmbH, Begisch Gladbach, Germany) was used to separate $CD133^+$ cells from $CD133^-$ cells. Cells were counted using a cell sorter (Z1™ Coulter Counter, Beckman Coulter, Brea, CA, USA). The culture became turbid in buffer at 350 μl/10^8 cells. The cells were labeled using the CD133 MicroBead Kit (MACS®; cat. no. 130-097-049; Miltenyi Biotec GmbH) containing CD133/1(AC133)-Biotin, and the CD133 MicroBead Kit (human; cat. no. 130-050-801; Miltenyi Biotec GmbH), containing an Fc receptor-blocking reagent, and anti-biotin MicroBeads in buffer. The solution was loaded onto the column and set in the magnetic field of the separator and the unlabeled ($CD133^-$) cells were extracted. The CD133-labeled ($CD133^+$) cells were eluted from the column with 1 ml autoMACS Rinsing Solution (cat. no. 130-091-222; Miltenyi Biotec GmbH). The separated $CD133^-$ and $CD133^+$ cells were counted.

miRNA extraction and polymerase chain reaction (PCR). Small RNA-enriched RNA (500 ng/μl) was isolated from the cells using the miRNeasy micro kit (Qiagen Inc., Valencia, CA, USA). Reverse transcription and quantitative PCR were performed using the RT^2 miRNA PCR Array human brain cancer (catalogue no. MIHS-108ZA-12; Qiagen Inc.), and an ABI PRISM 7000 sequence detection system (Applied Biosystems, Tokyo, Japan) was used under the following cycling conditions: 1 cycle at 95˚C for 15 min, followed by 40 cycles at 94˚C for 15 sec, 55˚C for 30 sec and 70˚C for 30 sec. Comparative Ct analysis ($2^{-\Delta\Delta CT}$) was used to identify a set of 86 mRNAs that were differentially expressed between irradiated and non-irradiated $CD133^+$ cells. Measurements were recorded in triplicate.

Fluorescent immunohistochemistry. $CD133^+$ cells were fixed in 10% formaldehyde for 1 h. These cells were incubated with an anti-PTEN antibody (1:100; Abcam, Cambridge, MA, USA) for 1 h at room temperature, followed by a secondary, TRITC-conjugated anti-rabbit immunoglobulin G antibody (A21428; Life Technologies, Carlsbad, CA, USA) and bisbenzimide H33342 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in a humid chamber at 37˚C for 30 min. Images were captured on a microscope with MetaXpress Image Analysis software (Molecular Devices, Sunnyvale, CA, USA). Staining for PTEN expression by AlexaFluor 594 was scored using a 0-3 scale. Specimens with scores of 0 or 1 (no or negligible nuclear staining in <0-30% of GSC) were considered immunonegative. Specimens that showed an intermediate (borderline) score of 2 (weak to moderate nuclear staining in <30-60% of GSCs) were considered equivocal. Specimens with a score 3 (strong complete nuclear staining in >60% of GSCs) were considered immunopositive.

Statistical analysis. Data were tested for significance using analysis of variance, performed using miScript miRNA PCR Array Data Analysis software (Qiagen Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of miRNA expression in 60-Gy-irradiated $CD133^+$ A172 cells. The 60-Gy-irradiated $CD133^+$ A172 cells showed significant differential expression in 18 miRNAs compared with the non-irradiated cells. The expression was increased in the 60-Gy-irradiated $CD133^+$ A172 cells in miR-16-5p by 5.97 times (P=0.0095), in miR-17-5p by 11.48 times (P=0.0054), in miR-19a-3p by 66.18 times (P=0.000023), in miR-19b-3p by 34.25 times (P=0.0023), in miR-21-5p by 4.99 times (P=0.045), in miR-24-3p by 4.97 times (P=0.024), in miR-27a-3p by 11.04 times (P=0.002), in miR-29a-3p by 11.75 times (P=0.0012), in miR-29c-3p by 16.46 times (P=0.014), in miR-96-5p by 16.68 times (P=0.028), in miR-101-3p by 12.53 times (P=0.005), in miR-130b-3p by 6.92 times (P=0.037), in miR-137 by 15.65 times (P=0.044), in miR-138-5p by 16.47 times (P=0.012), in miR-221-3p by 9.59 times (P=0.017), in miR-222-3p by 2.72 times (P=0.045) and in miR-425-5p by 3.6 times (P=0.039). The expression was decreased in the 60-Gy-irradiated $CD133^+$ A172 cells in miR-129-5p by 6.71 times (P=0.048) (Fig. 1).

Immunohistochemistry. Immunostaining for PTEN was positive in the non-irradiated A172 $CD133^+$ cells (positive rate, 80%; score, 3). The expression of PTEN was markedly decreased in the A172 $CD133^+$ cells following irradiation with 60 Gy (positive rate, 10%; score, 1) (Fig. 2).

Discussion

Significant differences were noted in the expression levels of 18 miRNAs between the 60-Gy-irradiated GSCs and the non-irradiated cells in the present study. The expression levels of 17 miRNAs (miR-16-5p, -17-5p, -19a-3p, -19b-3p, -21-5p, -24-3p, -27a-3p, -29a-3p, -29c-3p, -96-5p, -101-3p, -130b-3p, -137, -138-5p, -221-3p, -222-3p and -425-5p) were significantly higher, while that of miR-129-5p was significantly lower in the 60-Gy-irradiated GSCs compared with the non-irradiated cells. Of these, miR-17-5p, -19a-3p, -19b-3p, -21-5p, -130b-3p, -221-3p and -222-3p were previously shown to target PTEN,

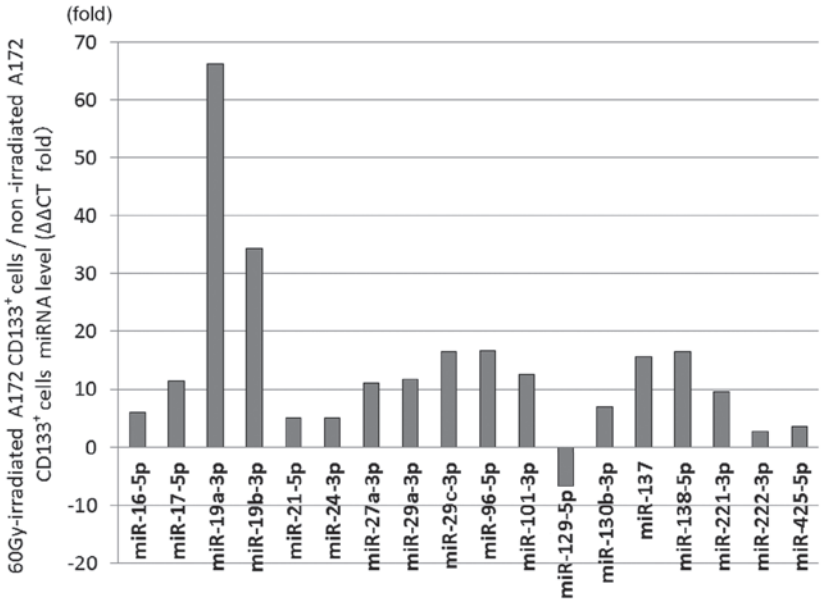

Figure 1. Analysis of microRNA (miRNA/miR) expression in 60 Gy-irradiated cluster of differentiation (CD)133+ A172 cells. The expression levels of 17 miRNAs were significantly higher, while that of miR-129-5p was significantly lower in the 60-Gy-irradiated glioblastoma stem-like cells than in the non-irradiated cells.

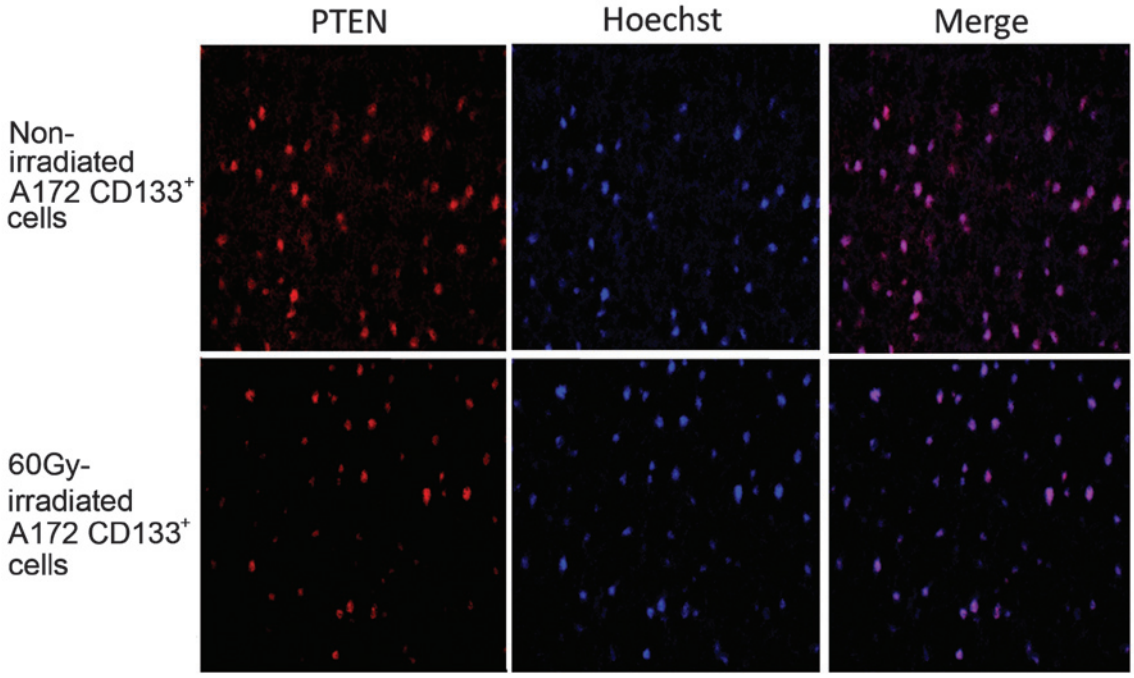

Figure 2. Immunostaining for phosphatase and tensin homolog deleted on chromosome 10 (PTEN) in non-irradiated and 60-Gy-irradiated A172 cluster of differentiation (CD)133+ cells. The nuceli were stained by Hoechst 33342 (blue) stain and PTEN by AlexaFluor 594 (Texas red). Images were captured at x200 magnification.

indicating that the regulation of PTEN expression through these miRNAs induces apoptosis in cultured brain tumor cells (16-18)

miRNA complementarily binds to a specific nucleotide in the 3'-UTR of mRNA, and inhibits target mRNA translation and protein expression. The inhibition of PTEN expression by the upregulated expression of miR-17-5p, -19a-3p and -19b-3p in malignant lymphomas, oligodendrocytes and oncogenic cells has been reported previously (19,20), and an inverse correlation was found between the expression of miR-130 and PTEN in osteosarcoma cell lines (21). A previous study showed that miR-21 acted together with miR-155 in a downstream signaling pathway in natural killer cell tumors and cell lines, and inhibited the expression of the target PTEN and PDCD4 (22). Furthermore, the increased expression of miR-221-3p and -222-3p inhibited that of PTEN in glioma cells (16,17). These findings suggest that the expression of miR-17-5p, -19a-3p, -19b-3p, -21-5p, -130b-3p, -221-3p and

-222-3p enhanced and inhibited the PTEN expression in the 60-Gy-irradiated GSCs in the present study. Immunostaining confirmed the localization of PTEN expression in the nucleus, and revealed that its expression was weaker in the 60-Gy-irradiated GSCs than in the non-irradiated cells. The presence of PTEN in the cytoplasm has been shown to inhibit the activation of Akt by dephosphorylating PIP_3, which induces cell apoptosis (23,24). However, PTEN has also been found to be strongly localized in the nuclei of undifferentiated cells in the resting G_0 phase, and to inhibit cell proliferation through an Akt-independent molecular mechanism (18,25,26). PTEN was expressed in the nuclei of the non-irradiated and 60-Gy-irradiated GSCs in the present study, suggesting that the proliferation of the GSCs was inhibited through an Akt-independent molecular mechanism, as were their self-differentiation and replication abilities, which are characteristic of GSCs. In addition, the expression of PTEN was weaker in the 60-Gy-irradiated GSCs than in the non-irradiated cells, which indicated that the inhibition of cell proliferation, self-differentiation and self-replication was weaker in the 60-Gy-irradiated GSCs.

PTEN was previously shown to suppress tumors through the Rb/E2F signaling pathway, and to promote Rb- and E2F-related apoptotic reactions (27), suggesting that the suppressed expression of PTEN in 60-Gy-irradiated GSCs leads to the resistance to apoptosis.

Although PTEN has been identified as a protein that induces apoptosis in cells, only a few treatments currently target the PTEN protein. The PTEN protein may be applied to treatments in order to induce apoptosis in irradiation-resistant GSCs.

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