Alteration of DNA damage signaling pathway profile in radiation-treated glioblastoma stem-like cells

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Abstract. The present study aimed to investigate the alteration of the DNA damage signaling pathway profile in radiation-treated glioblastoma stem-like cells (GSLCs), and also aimed to explore potential targets for overcoming glioblastoma radioresistance. Serum-free medium was used to isolate and culture GSLCs. Cell growth was detected using a cell counting kit-8 assay and cell sorting analysis was performed by flow cytometry. X-ray irradiation was produced by a Siemens-Primus linear accelerator. Reverse transcription-quantitative polymerase chain reaction (qPCR) was performed to investigate target genes. SPSS 15.0 was used for all statistical analyses. Human glioblastoma U251 and U87 cells were cultured in serum-free medium supplemented with epidermal growth factor and fibroblast growth factor 2, which constitutes tumor sphere medium, and demonstrated sphere formation, with significantly increased the proportion of $CD133^+$ and $Nestin^+$ cells, which are referred to as GSLCs. The present data revealed that treatment with 10 Gy X-ray radiation alters the expression profile of DNA damage-associated genes in GSLCs. The expression levels of 12 genes demonstrated a ≥2-fold increase in the irradiated U87 GSLCs compared with the untreated U87 GSLCs. Three genes, consisting of *XPA*, *RAD50* and *PPP1R15A*, were selected from the 12 genes by gene functional searching and qPCR confirmatory studies, as these genes were considered to be potential targets for overcoming radioresistance. The expression of *XPA, RAD50* and *PPP1R15A* is significantly increased in U87 and U251 radiation resistant GSLCs, indicating three potential targets for overcoming the radioresistance of GSLCs.

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Introduction

Glioblastoma (GBM) is considered to be the most aggressive and common type of primary malignant brain tumor in adult humans (1). With the administration of the standard treatment of maximal safe surgical resection followed by radiotherapy combined with concomitant and adjuvant temozolomide (TMZ) chemotherapy, newly diagnosed patients with GBM demonstrate an average survival time of only 12-14 months (2). Almost one-half of patients with GBM do not survive the first year subsequent to diagnosis (3-6).

It has previously been hypothesized that GBM initiation and recurrence is dependent upon a group of tumor stem cells that are resistant to current standard treatments (7,8). Certain studies have demonstrated that glioma stem cells (GSCs) are highly resistant to radiotherapy due to the enhanced checkpoint response to radiation (9). Additional investigation of the pathways involved in radioresistance is required.

In cancer cells, DNA damage may cause cell apoptosis, which is the purpose of chemotherapy or radiotherapy. Thus, the condition of DNA damage may affect radiosensitivity in GSCs (10). To investigate the alteration of the DNA damage signaling pathway in irradiated GSCs and to profile the genes of this pathway, the present study cultured the human glioblastoma U87 and U251 cell lines in serum-free medium (SFM) and obtained U87 or U251-glioblastoma stem-like cells (GSLCs). Irradiated U87 GSLCs were tested using gene chip polymerase chain reaction (PCR) array for the human DNA damage signaling pathway, and irradiated U251 GSLCs were used in quantitative PCR (qPCR) confirmatory studies.

Materials and methods

Cell culture. U87 and U251 human glioblastoma cell lines were purchased from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) supplemented with 1% Penicillin-Streptomycin-Glutamine (100X; Gibco Life Technologies, Carlsbad, CA, USA)

at 37˚C in a 5% CO_2 atmosphere. U87 and U251 GSLCs were induced from U87 and U251 cells, respectively, in SFM composed of DMEM/F12 (Gibco Life Technologies), 20 ng/ml basic fibroblast growth factor (FGF), 20 ng/ml epidermal growth factor (EGF; Gibco Life Technologies), 2 μg/ml heparin (Sigma-Aldrich, St. Louis, MO, USA) and B27 supplement (50X; Invitrogen, Carlsbad, CA, USA). The study was approved by the ethics committee of the Second Affiliated Hospital of Soochow University (Suzhou, China).

Cell growth. Cell growth analysis was performed using cell counting kit-8 (CCK-8) from Dojindo Laboratories (Kumamoto, Kumamoto, Japan), according to the manufacturer's instructions. Briefly, the cells were cultured at a density of 5,000 cells/well in 200 μl of culture medium and treated with 10 Gy X-ray radiation or left untreated. The cells were then cultured in a humidified incubator with a 5% CO_2 atmosphere. After 72 h, 20 μl CCK-8 solution was added to each well and the cells were incubated for 1 h in the incubator. The optical density value was determined by measuring the absorbance at 450 nm using an ELISA plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell sorting analysis by flow cytometry. Cells were cultured, collected and incubated with a phycoerythrin-labeled mouse anti-human CD133 IgG_1 monoclonal antibody (1:1,000; catalog no. 130-080-801; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or fluorescein isothiocyanate-labeled mouse anti-human Nestin IgG_1 monoclonal antibody (1:1,000; catalog no. MAB1259; R&D Systems, Inc., Minneapolis, MN, USA) for flow cytometry for 1 h at 37˚C. Subsequent to washing, the cells were analyzed by fluorescence-activated cell sorting (FACS) using a BD FACSAria Cell Sorter (BD Biosciences, San Jose, CA, USA).

X-ray irradiation. The cells were cultured in SFM and grown as GSLCs. The GSLCs were then exposed to 6 MV X-ray irradiation, produced by a Siemens-Primus linear accelerator (Siemens, Munich, Bavaria, Germany), at a dose rate of 2 Gy/min. The total dose administered was 10 Gy. The GSLCs were then harvested 72 h later for PCR array analysis or qPCR.

RNA isolation. The cells were cultured and harvested, and then added to 1 ml TRIzol reagent (Invitrogen) prior to being homogenized for 1 min at room temperature. Chloroform was added, and the sample was mixed with thermal agitation for 15 sec, followed by incubation at room temperature for 3 min and centrifugation at 14,000 x g for 15 min at 4˚C. The upper aqueous phase was collected, and the RNeasy Micro kit (Qiagen, Valencia, CA, USA) was used for the purification of total RNA from cell samples, according to the manufacturer's instructions.

Human DNA damage signaling pathway PCR array. The RNA samples were tested for the relative expression of genes involved in DNA damage signaling by the Human DNA Damage Signaling RT² Profiler™ PCR Array (SABiosciences, Frederick, MD, USA) according to the manufacturer's instructions. Briefly, the RNA was reverse transcribed to form cDNA. The samples were diluted in qPCR master mix

Table I. Sets of genes analyzed in the Human DNA Damage Signaling RT² Profiler™ polymerase chain reaction array.

Set	1	2	3	4	5	6	7	8	9	10	11	12
A	ABL1	ANKRD17	APEX1	ATM	ATR	ATRX	BRCA1	BTG2	CCNH	CDK7	CHEK1	CHEK2
B	CIB1	CIDEA	CRY1	DDB1	DDIT3	DMC1	ERCC1	ERCC2	EXO1	FANCG	FEN1	XRCC6
C	GADD45A	GADD45G	GML	GTF2H1	GTF2H2	GTSE1	HUS1	IGHMBP2	IP6K3	XRCC6BP1	LIG1	MAP2K6
D	MAPK12	MBD4	MLH1	MLH3	MNAT1	MPG	MRE11A	MSH2	MSH3	MUTYH	N4BP2	NBN
E	NTHL	OGG1	PCBP4	PCNA	AIFM1	PMS1	PMS2	PMS2L3	PNKP	PPP1R15A	PRKDC	RAD1
F	RAD17	RAD18	RAD21	RAD50	RAD51	RAD51L1	RAD9A	RBBP8	REV1	RPA1	SEMA4A	SESN1
G	SMC1A	SUMO1	TP53	TP73	TREX1	UNG	XPA	XPC	XRCC1	XRCC2	XRCC3	ZAK
H	B2M	HPRT1	RPL13A	GAPDH	ACTB	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

RTC, reverse transcription control; PPC, polymerase chain reaction positive control.

Table II. Genes that demonstrated a >2-fold increase in expression in irradiated cells compared with the untreated U87 glioblastoma stem-like cells.

Gene	Full name	Location	Fold change
BRCA1	Breast cancer 1, early onset	17q21-q24	-2.13
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	22q13.1	-3.11
GML	Glycosylphosphatidylinositol anchored molecule like	8q24.3	4.71
GTSE1	G-2 and S-phase expressed 1	22q13.2-q13.3	-3.24
IP6K3	Inositol hexakisphosphate kinase 3	6p21.31	2.19
N4BP2	NEDD4 binding protein 2	4p14	4.24
NBN	Nibrin	8q21-q24	-2.05
PPP1R15A	Protein phosphatase 1, regulatory subunit 15A	19q13.2	2.74
RAD50	RAD50 homolog (*S. cerevisiae*)	5q23-q31	2.42
SEMA4A	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	1q22	3.46
TREX1	Three prime repair exonuclease 1	3p21.31	2.34
XPA	Xeroderma pigmentosum, complementation group A	9q22.3	4.31

(RT2 SYBR Green; SABiosciences), according to the supplier's instructions, and pipetted into 96-well PCR array plates. qPCR was performed in technical duplicates (ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA, USA). Raw data from the real-time PCR was uploaded using the RT^2 Profiler PCR Array Data Analysis template (SABiosciences). A large number of gene expression analyses have been performed and the results published using the arrays and web-based automated RT^2 Profiler PCR Array Data Analysis method. The integrated web-based software package for the PCR array system automatically performed all comparative threshold cycle-based fold-change calculations from the uploaded data. For these calculations, the average expression of the reference gene β-actin was used for normalization of the data. Subsequent to normalization, the relative expression of each gene was averaged for the three samples in each cell group. Fold changes in average gene expression were expressed as the difference in expression of untreated U87 GSLCs compared with the difference in expression of radiation treated cells.

RT-qPCR. The miScript Reverse Transcription kit (Qiagen) was used to generate cDNA from RNA for qPCR. The cDNA template (100 ng) was used to perform RT-qPCR analyses for XPA, RAD50, PPP1R15A and α-tubulin using their specific forward primers and reverse primers, according to the manufacturer's instructions (Qiagen). The 5'-3' primer sequences were as follows: XPA forward, ATGTAAAAGCAGCCCCAAAGA, and reverse, TGGCAAATCAAAGTGGTTCATA (191 bp amplicon); RAD50 forward, GCGGAGTTTTGGAATAGAGGAC, and reverse, GAGCAACCTTGGGATCGTGT (185 bp amplicon); PPP1R15A forward, GATGGCATGTATGGTGAGCG, and reverse, GGTGTGATGGTGGATAAGAGAACT (208 bp amplicon); and α-tubulin forward, AGATCATTGACCTCGTGTTGGA, and reverse, ACCAGTTCCCCCACCAAAG (101 bp amplicon) (National Institutes of Health, Bethesda, MD, USA). α-tubulin was used as a control to normalize the levels of the genes. The StepOnePlus RT-PCR system (Applied Biosystems) was used to perform qPCR, using a hot start at 95˚C for 15 min, then denaturation at 95˚C for 15 sec, with annealing for 30 sec at 60˚C and extension for 30 sec at 72˚C for 40 cycles, followed by a melt curve analysis. Data analysis for the differences in gene expression between the control and radiation-treated cells was performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Gene expression was quantified using the following equation: $\Delta\Delta Ct = (Ct_{\text{radiation-treated sample}} - Ct_{\text{internal control}}) - (Ct_{\text{control sample}} - Ct_{\text{internal control}})$, where Ct is the mean threshold cycle from each group. The fold change was calculated as $2^{-\Delta\Delta Ct}$.

Statistical analysis. All data were expressed as the mean ± standard deviation and analyzed using one-way analysis of variance, followed by the Student-Newman-Keuls post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference. SPSS 15.0 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Serum-free culture induces U251 and U87 cells with GSC properties. U251 and U87 human glioblastoma cells were cultured in serum-free medium supplemented with EGF and FGF2, which comprises tumor sphere medium, and demonstrated sphere formation (Fig. 1C and D). Flow cytometry was used to score the proportion of $CD133^+$ and $Nestin^+$ cells in normal medium or serum-free medium cultured cells. As the results in Fig. 1E and F demonstrate, the proportion of $CD133^+$ and $Nestin^+$ cells were all significantly increased in U251 and U87 cells cultured in serum-free medium compared with the cells cultured in normal medium. Since CD133 and Nestin are the most widely used markers to identify GSCs,

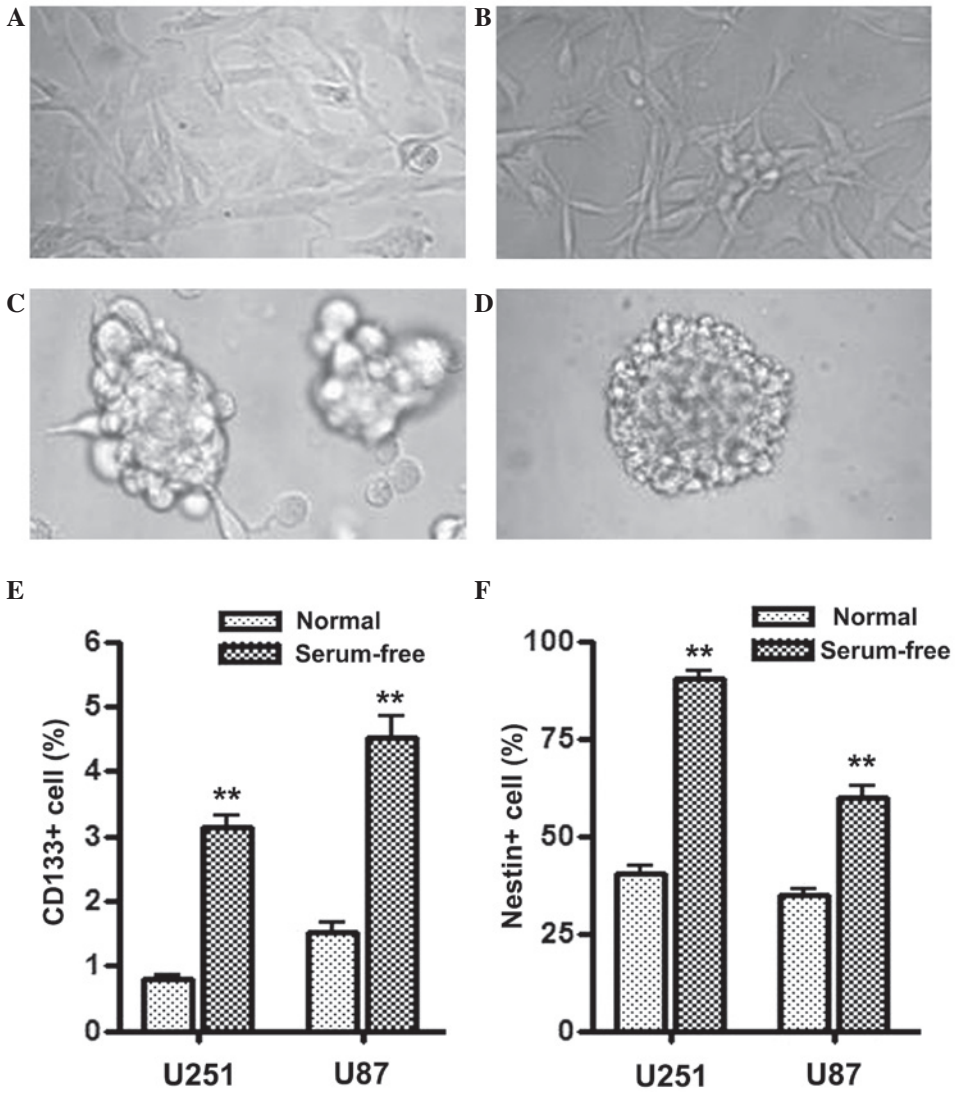

Figure 1. Culturing U251 and U87 cells in serum-free medium induces cell sphere formation. (A) U251 and (B) U87 cells were cultured in normal medium. (C) U251 and (D) U87 cells were cultured in serum-free medium supplemented with epidermal growth factor and fibroblast growth factor 2, which constituted tumor sphere medium, and demonstrated sphere formation. (E and F) The proportion of $CD133^+$ cells or $Nestin^+$ cells was significantly increased when the cells were cultured in serum-free medium ($^{**}P<0.01$). Normal, cells cultured in normal medium; Serum-free, cells cultured in serum-free medium; CD133, cluster of differentitation 133.

the present results indicate that U251 and U87 cells cultured in tumor sphere medium became enriched in properties of GSCs. In the current study, these sphere formatted cells cultured in serum-free medium are referred to as GSLCs.

Radiation treatment alters the expression profile of DNA damage-associated genes in GSLCs. The U87 GSLCs were cultured to a population of 2-5x10^6 cells and treated with 10 Gy radiation. After 72 h, the cells were harvested. Cell growth was detected using a CCK-8 kit, which revealed that 10 Gy radiation induced no cell growth suppression, indicating the radioresistance of U87 GSLCs (Fig. 2). The detection of the expression profiles of DNA damage-associated genes in cells were performed using DNA damage signaling pathway chip PCR array, which profiled the histone modification status, also termed histone code, of 90 genes involved in DNA damage signaling pathways. The detected genes are listed in Table I, with H7-9 acting as the reverse transcription control and H10-12 acting as the PCR positive control. Fig. 3A reports the alteration of the expression profile of DNA damage-associated genes. Gene expression levels that were increased ≥2-fold in the irradiated U87 GSLCs compared with the untreated U87 GSLCs are listed in Table II.

Confirmatory studies by TaqMan qPCR. To confirm the results of the PCR array, the RT-qPCR assay was performed using U251 GSLCs to assess the expression of the *XPA*, *RAD50* and *PPP1R15A* genes. The expression of these genes increased >2-fold and the genes were associated with radiation-induced DNA damage, as determined by gene functional searching from Entrez Gene and the published literature in PubMed Central (National Center for Biotechnology Information, Bethesda, MD, USA). To detect the genes, the U251 GSLCs were cultured to a population of 2-5x10^6 cells and treated with 10 Gy radiation. After 72 h, the cells were respectively harvested and the qPCR was performed, as well as the cell growth detection (Fig. 2). As the results in Fig. 3B demonstrate, after 72 h the expression of all three genes had significantly increased ($P<0.01$).

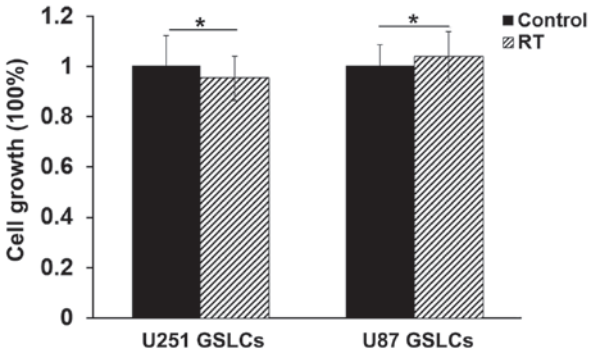

Figure 2. U251 and U87 GSLCs were resistant to 10 Gy X-ray radiation treatment. Cell growth was detected using a cell counting kit-8. U251 or U87 glioblastoma stem-like cells were treated with 10 Gy X-ray radiation or remained untreated. Cell growth was detected 72 h post-irradiation. The data revealed no differences between the irradiated and untreated cells (*P>0.05). Control, non-treated cells; RT, radiation treated cells.

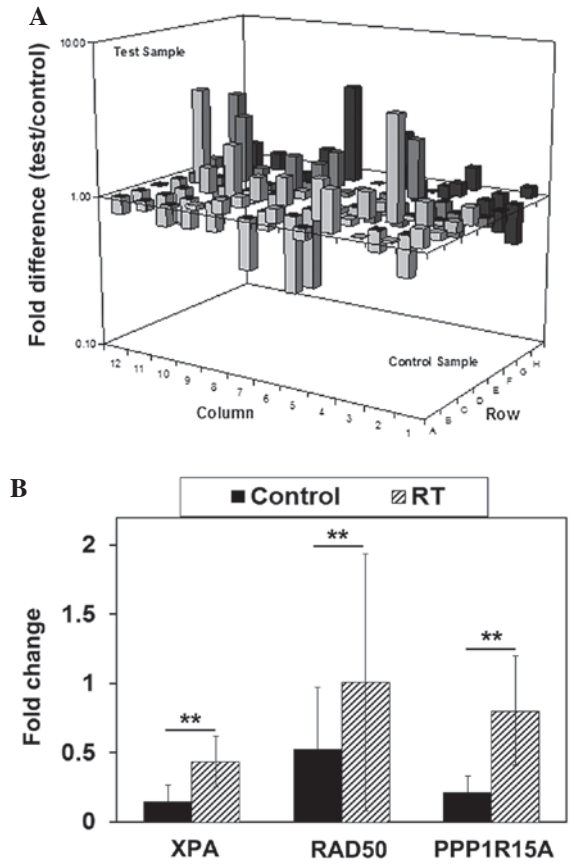

Figure 3. (A) Alteration in the expression profile of DNA damage signaling genes in U87 GSLCs following 10 Gy X-ray radiation. (B) The expression levels of the *XPA*, *RAD50* and *PPP1R15A* genes were significantly increased (**P<0.01) in U251 GSLCs following 10 Gy X-ray radiation, demonstrating a 1.98-fold increase for *XPA*, 1.67-fold increase for *RAD50* and 2.78-fold increase for *PPP1R15A*. GSLCs, glioblastoma stem-like cells; Control, non-treated cells; RT, radiation treated cells.

Discussion

Cancer stem cells are considered to be a subpopulation of cancer cells that is highly enriched with tumorigenic potential (9). GSCs have been found to demonstrate improved survival subsequent to irradiation and chemotherapy and contribute to the recurrence of GBM (11). In addition, GSCs frequently reside in perivascular and hypoxic regions, actively promoting angiogenesis and facilitating the survival of cancer cells in harsh environments (12). The presence of GSCs was considered to be the origin of the difficulty in treating GBM. Previously, GSCs were reported to isolate, in the form of spheres, from glioma tissues cultured in SFM containing the stem cell mitogens EGF and FGF, which is the same method used to isolate neural stem cells from brain tissue (13). In the present study, GSLCs were isolated from the human GBM U87 and U251 cell lines using the previously described GSCs-isolation SFM method, and the GSLCs were characterized by sphere formation ability and positivity for CD133 or other putative stem markers such as Sox2 and Nestin (13). Flow cytometry was used to score the proportion of $CD133^+$ and $Nestin^+$ cells, and it was found that each of these proportions was significantly increased in GSLCs cultured in serum-free medium compared with those cultured in normal medium. Since CD133 and Nestin are considered to be valuable stem cell-specific markers for determining the clinical outcome of glioma patients, these markers were used in the present study for cell identification (14).

The identified GSLCs isolated from the U87 and U251 cells were subsequently used in additional investigations. The cells were irradiated with 10 Gy X-rays after 72 h, and the U87 and U251 GSLCs demonstrated radioresistance with no cell growth suppression. The DNA damage signaling pathway chip PCR array, which profiled the histone modification status, also termed the histone code, of 90 genes involved in DNA damage signaling pathways, was performed on the irradiated U87 GSLCs. According to the PCR array results, the expression of three genes, consisting of *XPA*, *RAD50* and *PPP1R15A*, was increased >2-fold and these genes were involved in radiation-associated DNA damage. These genes were selected to undergo qPCR confirmatory investigation in U251 GSLCs.

XPA encodes a zinc finger protein involved in DNA excision repair. The XPA protein is a component of the nucleotide excision repair (NER) complex, which is responsible for the repair of ultraviolet (UV) radiation-induced photoproducts and DNA adducts induced by chemical carcinogens (15). The XPA protein is one of eight factors that were found to be deficient in XP disorders, which are characterized by an increased sensitivity to UV radiation and a predisposition to development of skin cancers (16). Functionally, XPA is considered to play roles in verifying DNA damage, stabilizing repair intermediates, and recruiting other NER factors to the damaged DNA (17).

The protein encoded by *RAD50* is involved in DNA double-strand break repair. The RAD50 protein forms a complex with Mre11/NBS1 to form the Mre11/RAD50/NBS1 (MRN) complex (18). The protein complex binds to DNA and exhibits numerous enzymatic activities that are required for the non-homologous joining of DNA ends. The MRN complex is a conserved protein complex that plays a key role in the response to DNA damage (19). Kuroda1 *et al* (20) demonstrated that degradation of MRN complex results in the inability to repair DNA double-strand breaks (DSB) and leads to radiosensitization, which indicates that RAD50 plays a key role in radioresistance.

PPP1R15A, also termed *GADD34*, is a member of a group of genes that demonstrate increased transcript levels following

stressful growth arrest conditions and treatment with DNA-damaging agents (21). The expression of the PPP1R15A protein increases in response to medium depletion or DNA damage, heat shock and several other treatments that induce apoptosis (22). Overexpression of *PPP1R15A* enhances the apoptotic response to DNA damage following ionizing radiation (23). The transcriptional induction of *PPP1R15A* is strongly associated with apoptosis (24).

The present data obtained from RT-qPCR confirmatory studies in U251 GSLCs revealed that 72 h after the administration of 10 Gy radiation, the expression of all three genes was significantly increased, with a 1.98-fold increase in *XPA* expression, 1.67-fold increase in *RAD50* expression and 2.78-fold increase in *PPP1R15A* expression. The upregulation of the three genes in irradiated GSLCs after 72 h may indicate the alteration of DNA damage repair responses. The induction of *XPA*, *RAD50* and *PPP1R15A* by X-ray treatment may confer radioresistance in GSLCs. In conclusion, the present study revealed the alteration of the DNA damage signaling pathway profile in radiation treated GSLCs. Out of the genes that demonstrated a significant increase in expression, the *XPA*, *RAD50* and *PPP1R15A* radiation-associated genes were selected for additional study. The expression of these three genes was significantly increased in U87 and U251 radiation-resistant GSLCs, indicating three potential targets for overcoming GSLCs radioresistance. It is necessary to investigate these genes separately in radiation treated GSCs.

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