Radiosensitivity in HeLa cervical cancer cells overexpressing glutathione S-transferase π 1

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Received May 15, 2014; Accepted February 10, 2015

DOI: 10.3892/ol.2015.3394

Abstract. The aims of the present study were to investigate the effect of overexpressed exogenous glutathione S-transferase π 1 (*GSTP1*) gene on the radiosensitivity of the HeLa human cervical cancer cell line and conduct a preliminarily investigation into the underlying mechanisms of the effect. The full-length sequence of human *GSTP1* was obtained by performing a polymerase chain reaction (PCR) using primers based on the GenBank sequence of *GSTP1*. Subsequently, the gene was cloned into a recombinant eukaryotic expression plasmid, and the resulting construct was confirmed by restriction analysis and DNA sequencing. A HeLa cell line that was stably expressing high levels of *GSTP1* was obtained through stable transfection of the constructed plasmids using lipofectamine and screening for G418 resistance, as demonstrated by reverse transcription-PCR. Using the transfected HeLa cells, a colony formation assay was conducted to detect the influence of *GSTP1* overexpression on the cell radiosensitivity. Furthermore, flow cytometry was used to investigate the effect of *GSTP1* overexpression on cell cycle progression, with the protein expression levels of the cell cycle regulating factor cyclin B1 detected using western blot analysis. Colony formation and G_2/M phase arrest in the *GSTP1*-expressing cells were significantly increased compared with the control group ($P<0.01$). In addition, the expression of cyclin B1 was significantly reduced in the *GSTP1*-expressing cells. These results demonstrated that increased expression of *GSTP1* inhibits radiosensitivity in HeLa cells. The mechanism underlying this effect may be associated with the ability of the GSTP1 protein to reduce cyclin B1 expression, resulting in significant G_2/M phase arrest.

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Key words: glutathione S-transferase π 1, human cervical cancer, cell cycle, radiosensitivity

Introduction

In consideration of the increase in tumor incidence and mortality rates, numerous studies are focusing on the identification of practical and effective cancer treatment strategies (1). Currently, the predominant treatment strategies for the majority of tumors include surgery, chemotherapy and radiotherapy; however, an effective strategy for the treatment of cancer remains to be established (2).

Tumor radiotherapy has been under development for >100 years and has become an important method of local treatment for patients in different clinical stages of cancer (3). Recent studies have demonstrated that 70% of cancer patients require adjuvant radiotherapy during their treatment course (3,4). For specific tumor types, including nasopharyngeal, esophageal and prostate cancer tumors, radiotherapy may even replace surgical treatment (3). Thus, radiotherapy is important in the treatment of cancer. However, for specific cases of advanced or metastasized tumors with low radiosensitivity, the increased dose of radiation required may damage the surrounding healthy tissues and organs (3). Therefore, the identification of novel methods to enhance tumor cell radiosensitivity has become a recent focus of medical radiation research (4).

Glutamine S-transferases (GSTs) are an most important enzyme family involved in the redox reaction and are key in the protection of cells from toxins and carcinogens. For instance, GST π 1 (GSTP1) is a member of the GST family that is involved in detoxification (5), with recent studies identifying GSTP1 as an important marker of diagnosis, prognosis and chemotherapy resistance in certain tumors (6-9). Therefore, the aims of the present study were to investigate the effect of *GSTP1* gene overexpression on the radiosensitivity of the HeLa human cervical cancer cell line, to preliminarily investigate the underlying mechanisms of these effects and to provide an experimental foundation for improving the effects of clinical radiotherapy in the future.

Materials and methods

Materials. The HeLa human cervical carcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), Dulbecco's modified Eagle's

medium (DMEM) and newborn calf serum was obtained from Gibco Life Technologies (Carlsbad, CA, USA), G418 from Inalco Pharmaceuticals (San Luis Obispo, CA, USA), TRIzol reagent and lipofectamine from Invitrogen Life Technologies (Carlsbad, CA, USA), the anti-cyclin B1 antibody from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and the polymerase chain reaction (PCR) instrument and Gel Doc 2000 gel imager from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Generating stably transfected cell lines. HeLa cells were cultured in DMEM medium containing 10% newborn calf serum. Prior to transfection, a total of $1.2x10^6$ cells/well were seeded in a 35-mm petri dish for 48 h. Human full-length *GSTP1* cDNA (Genecopoeia, Rockville, MD, USA) was directionally cloned into the eukaryotic expression vector, pcDNA3 (Genecopoeia). The construct was subsequently confirmed by restriction endonuclease (Corning Life Sciences-Axygen Inc., Union City, CA, USA) and DNA sequence analysis. The pcDNA3/*GSTP1* construct or the empty pcDNA3 vector (negative control) was then transfected into the HeLa cells using a liposome-mediated method (10) and cultured in medium containing 500 μg/ml G418 for four weeks at 37˚C for selection. Following transfection, reverse transcription (RT)-PCR was performed to detect the *GSTP1* mRNA expression levels. HeLa cells transfected with the empty pcDNA3 plasmid were termed as HeLa/Neo cells and those transfected with pcDNA3/*GSTP1* were termed as HeLa/*GSTP1* cells. Prior to irradiation, all the cells were transferred to normal culture medium for two days to avoid interference from G418.

Clone formation assay. HeLa cells were seeded into flasks at a concentration of $1x10^5$/ml. After a 12-h incubation period, the cells were synchronized by replacing the medium with serum-free DMEM and cultured for 24 h before irradiation. Radiation, at a dose rate of 200 cGy/min, a source-skin distance of 100 cm and a dose gradient of 0, 2, 4, 6 and 8 Gy, was then applied to the cells. Following irradiation, the cells were cultured in normal medium for two weeks, fixed with methanol for 30 min and stained in Giemsa (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Subsequent to the removal of excess dye, the number of colonies containing >25 cells were counted under a microscope to determine the rate of colony formation and calculate the survival curves.

Flow cytometry. Following synchronization, the HeLa cells were digested using EDTA-free trypsin (Sigma-Aldrich), washed twice in phosphate-buffered saline (PBS), fixed in 70% ethanol for 12 h and filtered through a 300 μm mesh nylon screen (Sangon Biotechnology Co., Ltd., Shanghai, China). The cells were then stained with 200 μl propidium iodide dye (100 μg/ml; Sigma-Aldrich) for 30 min in the dark prior to cell cycle analysis using an EPICS XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA).

RT-PCR. Total RNA was extracted from the HeLa cells using TRIzol reagent, according to the manufacturer's instructions, and RNA purity was determined using an ultraviolet spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The Transcriptor First Strand cDNA Synthesis kit, which was obtained from Roche Diagnostics GmbH (Mannheim, Germany) was used for the experiments. Briefly, 1 μl total RNA from each group was added to 1 μl of oligo (dT) and 10 μl diethylpyrocarbonate-treated water, and incubated in a water bath at 70˚C for 5 min, prior to cooling on ice for 30 sec. Subsequently, 4 μl 5X reaction solution, 1 μl RNase inhibitor (20 U/μl) and 2 μl deoxynucleoside triphosphate (10 mmol/l) were added to the reaction, which was heated in a water bath at 37˚C for 5 min. Following the addition of 1 μl Moloney-murine leukemia virus reverse transcriptase (20 μg/μl), reverse transcription was allowed to proceed for 1 h at 37 ˚C before the reaction was inactivated by heat treatment for 10 min at 70˚C.

Primers for *GSTP1* (Genebank accession no. U21689.1) and *GAPDH* (Genebank accession no. NH-017008) were designed against their GenBank sequences using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA). The primer sequences for *GSTP1* were as follows: Forward, 5'-ATTAACCCTCACTA-AAGGGAGATATGGTGAAGGAATGATGGGGT-3', and reverse, 5'-TAATACGACTCACTATAGGGGGATCTTGGG CCGGGCACTGA-3'; the amplified product was 650 bp. The primer sequences for *GADPH* were as follows: Forward, 5'-CAACTACATGGTCTACATGTTCC-3', and reverse, 5'-CAACCTGGTCAGTGTAG-3'; the amplicon was 700 bp. The PCR reaction conditions were as follows: Reverse transcription at 48˚C for 45 min; inactivation and denaturation for 2 min at 94˚C; 35 cycles of annealing at 58˚C for 45 sec and elongation at 72˚C for 45 sec; and extension at 68˚C for 7 min. Finally, the PCR product was electrophoresed through a 2% agarose gel and images were captured using the Gel Doc 2000 gel imager (Bio-Rad Laboratories, Inc.).

Western blot analysis. HeLa cells were collected and lysed using radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The total protein concentration was determined using the Lowry method, with 100 μg protein separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes with 5% skimmed milk powder.

Following addition of the monoclonal mouse anti-human anti-β-actin (1:5,000; cat. no. sc-24979; Santa Cruz Biotechnology, Inc.) and monoclonal mouse anti-human cyclin B1 antibodies (1:1,000; cat. no. sc-166757; Santa Cruz Biotechnology, Inc.), the blots were incubated overnight at a temperature of 4˚C on an oscillating platform and then washed three times in PBS/Tween 20 for a total of 15 min. Finally, the blots were incubated with a monoclonal mouse anti-human horseradish peroxidase-labeled IgG secondary antibody (1:5,000; cat. no. sc-9969; Santa Cruz Biotechnology, Inc.) and developed using a LightShift Chemiluminescent EMSA Kit (Sangon Biotechnology Co., Ltd.).

Statistical analysis. All the experiments were repeated three times. The independent samples t-test and correlation analysis were performed and SAS statistical software (version 8.0; SAS Institute. Inc., Cary, NC, USA) was used to analyze the results. $P<0.01$ was considered to indicate a statistically significant difference.

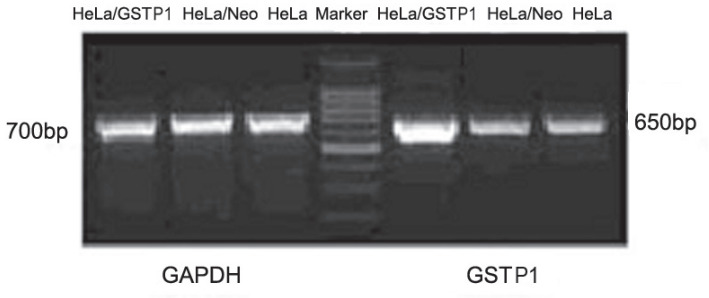

Figure 1. *GSTP1* expression in the three groups of HeLa cells. GSTP1, glutathione S-transferase π 1.

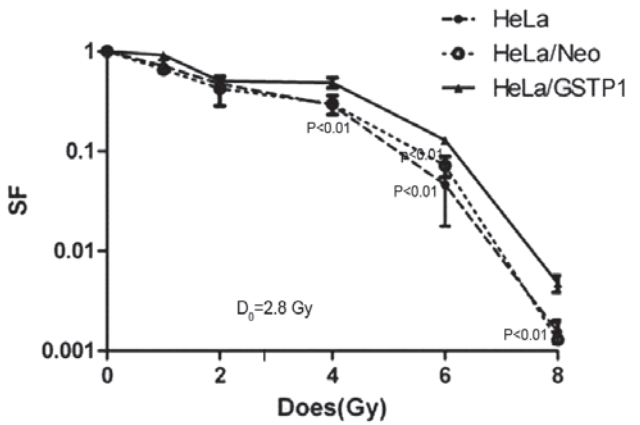

Figure 2. Survival curves for the three groups of HeLa cells following irradiation. GST, glutathione S-transferase π 1; SF, survival function.

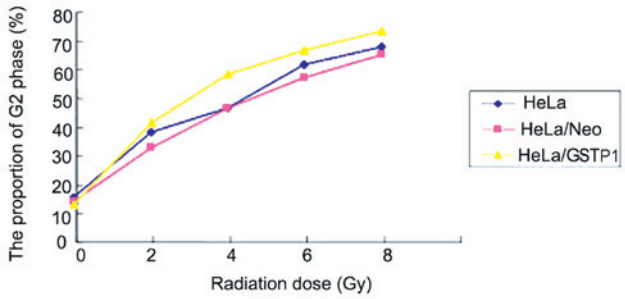

Figure 3. G_2 phase arrest following irradiation of the three groups of HeLa cells. GST, glutathione S-transferase π 1.

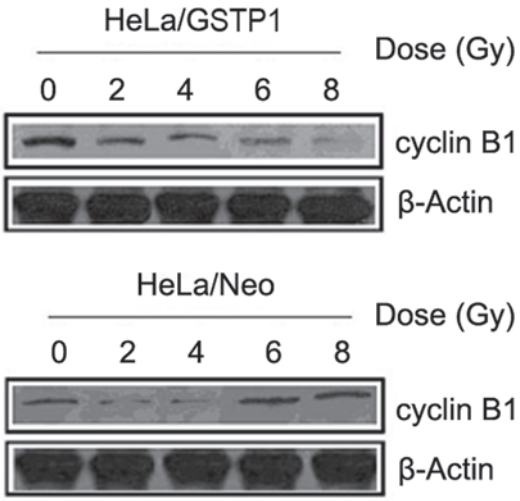

Figure 4. Expression of cyclin B1 protein in two groups of HeLa cells following irradiation. GST, glutathione S-transferase π 1.

Results

High GSTP1 gene expression in stably transfected HeLa cell lines. HeLa cells stably transfected with *GSTP1* and empty plasmid were successfully established, and their *GSTP1* mRNA expression levels were determined using RT-PCR. *GSTP1* mRNA expression was markedly higher in the HeLa/*GSTP1* cells compared with the non-transfected HeLa cells or the cells transfected with the empty vector (HeLa/Neo cells), as indicated in Fig. 1. By contrast, *GSTP1* mRNA expression in the HeLa/Neo cells was almost equivalent to the expression in the normal HeLa cells. The results indicated that the *GSTP1* recombinant plasmid was successfully constructed and highly expressed in HeLa cells, obtaining the HeLa/*GSTP1* cells.

GSTP1 gene overexpression reduces radiosensitivity in HeLa cells. As indicated in Fig. 2, the administration of increasing doses of radiation resulted in a dose-dependent downward trend in the cell survival rate of the non-transfected HeLa, HeLa/Neo and the HeLa/*GSTP1* cells. In particular, the survival rate was significantly higher in the HeLa/*GSTP1* cells irradiated with 2.8 Gy compared with the control group (P<0.01), with the HeLa/*GSTP1* cells demonstrating radiation resistance. Furthermore, the D_0 value (incremental dose required for reducing the fraction of colonies to 37%, indicative of single event killing) of the HeLa and HeLa/Neo cells was ~2.4 Gy; however, the D_0 value was 2.8 Gy in the HeLa/*GSTP1* cells, demonstrating a statistically significant difference (P<0.01). These experimental data indicated that the radiosensitivity in HeLa cells may be reduced by increasing the expression of *GSTP1*.

GSTP1 overexpression alters cell cycle progression in HeLa cells. As indicated in Fig. 3, the three groups of HeLa cells were treated with an X-ray radiation dose gradient of 0, 2, 4, 6 and 8 Gy. The results indicated that G_2 phase arrest occurred in a dose-dependent manner in the three groups of cells; however, G_2 arrest was more evident in the HeLa/*GSTP1* cells compared with the control group. The proportion of cells in the G2 phase was significantly higher in the HeLa/GSTP1 cells

when compared with the negative control group (HeLa/Neo) ($P<0.01$).

Impact of GSTP1 overexpression on cyclin B1 protein expression levels. Since cell cycle progression is affected by radiation, the expression of the cell cycle regulation protein cyclin B1 was detected by western blot analysis. As indicated in Fig. 4, the protein expression of cyclin B1 in the HeLa/*GSTP1* cells markedly decreased with an increasing radiation dose, while in the control group, cyclin B1 expression was unchanged.

Discussion

The underlying mechanisms that cause certain tumors to exhibit low radiosensitivity remain unclear. A previous study identified that various growth factors, including the insulin-like growth factor-1 receptor and epidermal growth factor, are involved in radiosensitivity (11). In addition, nuclear expression of anti-tumor genes or oncogenes, such as *p53* and B-cell lymphoma-2, may alter radiosensitivity in cells (12). Therefore, genes that affect the cell cycle, apoptosis and genomic stability may also affect the radiosensitivity of tumor cells (13-15).

The majority of studies have reported that G_2/M phase arrest is negatively correlated with cell radiosensitivity, with significant radiation resistance occurring in the S phase or in G_2/M phase arrest (16,17). Particularly in the G_2 phase, longer duration of the cell cycle arrest results in more frequent occurrence of potentially lethal DNA damage repair. Therefore, cell survival eventually increases as a function of radiation resistance (15). The results of the present study demonstrated that *GSTP1* may induce G_2 phase arrest by regulating the expression of the cyclin B1 cell cycle protein and, thus, reducing the radiosensitivity of HeLa cervical cancer cells.

Members of the GST super-gene family function in cell protection, storage, binding, transport and detoxification. In addition, overexpression of the isoenzyme, GSTP1, is closely associated with tumor occurrence; therefore, increased *GSTP1* expression may be used in the diagnosis, prognosis and determination of chemotherapy resistance in a variety of tumor tissues (18).

In conclusion, only a limited number of studies have thus far been conducted regarding the effect of *GSTP1* expression on tumor radiosensitivity (15,19,20). However, combined with the existing data on *GSTP1*, the present study hypothesized that *GSTP1,* as well as acting as a marker for diagnosis, prognosis and chemotherapy resistance, may serve as an important therapeutic agent in the treatment of various tumors.

Acknowledgements

The present study was supported by grants from the National Science Foundation of China (nos. 81072175, 81372854 and 81102010) and the Shanghai Committee of Science and Technology, China (no. 13NM1401504).

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