Original Article

GSTP1 arrests bladder cancer T24 cells in G0/G1 phase and up-regulates p21 expression

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Received July 5, 2014; Accepted August 16, 2014; Epub September 15, 2014; Published September 30, 2014

Abstract: Objective: GSTP1 over-expression was introduced into human bladder cancer T24 cells via the lentivirus system. The influence of GSTP1 on the proliferation and cell cycle of T24 cells as well as the potential mechanisms was investigated. Methods: The lentiviral vector GSTP1-pWPXL was constructed and transfected into T24 cells in the presence of Lipofectamine 2000. CCK8 assay and colony formation test were performed to explore the impact of GSTP1 on the proliferation of T24 cells. Ollowing PI staining, flow cytometry was done to detect the proportion of T24 cells in different phases. Western blot assay was conducted to detect the protein p21 expression. Results: When compared with control group, T24 cells with GSTP1 over-expression showed significant reduction in cell proliferation (P < 0.01) and they were arrested in G0/G1 phase. Western blot assay indicated that the p21 protein expression in T24 cells with GSTP1 over-expression was significantly higher than that in control group. Conclusion: GSTP1 may inhibit the proliferation of T24 cells and arrest these cells in G0/G1 phase, which may be ascribed to the up-regulated expression of p21.

Keywords: Bladder cancer, GSTP1, cell proliferation, cell cycle, p2

Introduction

Glutathione-S-transferases (GSTs) are a group of isoenzymes with extensive functions and can catalyze the binding of hydrophobic electrophilic compounds to reduced glutathione (GSH) making the products easy to secret; GSTs can also serve as binding proteins to bind to several hydrophobic compounds with high affinity (hormones, drugs and carcinogens) and transport them; they may also prevent lipid peroxidation. Since GSTs play important roles in metabolizing toxic compounds and protecting cells against toxic chemicals, they can protect normal cells against the damage induced by cancer-promoting factors and inhibit the cell canceration play crucial roles in antimutagenesis and anti-tumor. However, these effects may also cause the resistance of cancer cells to chemotherapeutics [1].

Available studies have shown that, of GST superfamily, GSTP1 is widely distributed in human tumors. GSTP1 gene is mapped to chromosome 11q13 with a full length of about 3 kb and contains 6 introns and 7 exons encoding 210 amino acids. GSTP1 has a high expression in the human malignancies derived from epithelium (such as gastrointestinal cancer, esophageal cancer, lung cancer, bladder cancer, thyroid cancer and breast cancer). To date, no studies have confirmed that malignancies except for non-Hodgkin’s lymphoma have no GSTP1 expression [2]. When compared with normal tissues, GSTP1 expression can be classified as three categories: increase (GSTP1 thus serves as a marker of cancers), reduction, and no change [3]. Thus, we speculate that GSTP1 play different roles in the cancerogenesis in distinct cancers.

Bladder cancer is the second most common cancer of the reproductive system second to prostate cancer. Its prevalence ranks 5th in Europe, 4th in USA and 8th in China. The incidence of bladder cancer in males is 3-4 times
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of that in females. Smoking and exposure to carcinogens are the most common risk factors for bladder cancer. Bladder cancer in more than 90% of patients is derived from urothelium, 5%-10% of bladder cancer is squamous cell carcinoma, 2%-3% is adenocarcinoma and about 70%-80% is superficial bladder cancer (SBC) [4].

To explore the role of GSTP1 gene in the occurrence and development of bladder cancer, this study was conducted to induce GSTP1 over-expression in human bladder cancer T24 cells, and the proliferation and cell cycle were detected in these cells, aiming to investigate the importance of GSTP1 in the evolution of bladder cancer.

Materials and methods

Materials

Human bladder cancer T-24 cells (Cell Bank of Chinese Academy of Science in Shanghai), lentiviral plasmids pWPXL, pA2 and pMDG2 kindly provided by Doctor Didier in the University of Geneva, High Purity Plasmid Miniprep Kit (TIANGEN; C1015-05), DMEM high glucose (GIBCO; C11995500BT), fetal bovine serum (FBS; Corille; C1015-05), CCK-8 kit (Shanghai DOJI-NDO LABORATORIES; CK04), lipofectamine 2000 transfection kit (Cat No. 11668-019; Invitrogen), GSTP1 monoclonal antibody (mouse; Cat No. sc-66000), β-actin monoclonal antibody (mouse; Cat No. sc-47778; Santa Cruz), p21 monoclonal antibody (mouse; Cat No. 2947s; CST), goat anti-mouse secondary antibody (Cat No. 31431; Thermo) and ECL luminescent substrate kit (Cat No. 34075; Pierce) were used in the present study.

Cell culture

T24 cells were maintained in DMEM containing 10% FBS at 37°C in a humidified environment with 5% CO₂. Cells in logarithmic growth phase were used in following experiments.

Construction of GSTP1 over-expressing lentivirus

The mRNA of GSTP1 was obtained from NCBI GenBank (NM_000852) and served as a template for the design of primers with restriction sites which were synthesized in Invitrogen. The primers were as follows: 5'-CGCGGATCCATGGCCCTACACCCTGGTGGT-3' (forward), 5'-CGGGAATTCTCAGCTTTCCCTGGTGCCATT-3' (reverse). The cDNA of T24 cells served as a template for PCR. A purification kit was used for retrieval of products after PCR (length: 651 bp). The target gene was digested with BamH I and EcoR I and the resultant products were connected into lentivirus pWPXL (after digestion with BamH I and EcoR I) in the presence of T4 DNA ligase at 16°C overnight. The products were used to infect competent DH5α cells which were then incubated on LB agar plates containing 100 μg/mL ampicillin at 37°C overnight. Single colony was harvested and cultured on LB agar plates for expansion at 37°C under a continuous shaking condition. Plasmids were extracted with a kit, and DNA concentration was determined by UV spectrophotometry. After digestion with BamH I and EcoR I, products were subjected to 1% agarose gel electrophoresis, followed by identification by sequencing.

Preparation of T24 cells with stable GSTP1 over-expression

293 T cells in logarithmic growth phase were seeded into 60 mm dishes and maintained for 24 h. When the cell confluence reached 70-80% on the second day, Transfer vector (pWPXL; 6 μg), packaging plasmid (pA2; 3 μg), envelope plasmid (pMDG2; 1.2 μg) and DMEM (2 ml) were added to a centrifuge tube, and 20 μl of Lipofectamine2000 and 2 ml of DMEM were added to another centrifuge tube. Following incubation of both mixtures for 5 min, they were mixed and allowed to stay at room temperature for 20-25 min. Then, mixture containing plasmids and Lipofectamine2000 was added to 293 T cells. After transfection for 48 h, the medium was harvested and centrifuged at room temperature for 5 min at 3000 rpm/min. The virus solution was collected after filtering through a 0.45 μm filter. One day before transfection, T24 cells were seeded into 35 mm dishes and cultured for 24 h. When the cell confluence reached 70-80%, the medium was refreshed and polybrene was added at a final concentration of 6 μg/ml. About 30 min later, the lentivirus solution (200 μl) following packaging was added, followed by infection for 12 h. Then, the medium was refreshed with complete medium, followed by further incubation for 14 at 37°C in an environment with 5% CO₂. Total
Figure 1. Identification of GSTP1-pWPXL following digestion with BamH I and EcoR I and subsequent sequencing. STP1-pWPXL was digested with BamH I and EcoR I, and products were subjected to 1% agarose gel electrophoresis. A protein band was observed between 750 bp and 500 bp (actual length: 651 bp).
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proteins were extracted from these cells for western blot assay for identification.

Detection of cell proliferation

Cells in control group and GSTP1 group were digested with 0.25% trypsin at room temperature and single cell suspension was prepared with DMEM containing 10% FBS. Then, these cells were seeded into 96-well plates at 1000 cells/well and incubated at 37°C in an environment with 5% CO₂ for 7 days. After 1 day, 3 days, 5 days, 7 days, 100 µL CCK-8 solution was added to each well and the absorbance at 450 nm was determined by a microplate reader (n = 3).

Colony formation test

Cells in control group and GSTP1 group were digested with 0.25% trypsin at room temperature and single cell suspension was prepared with DMEM containing 10% FBS. Then, these cells were seeded into 96-well plates at 1000 cells/well and incubated at 37°C in an environment with 5% CO₂. On days 1, 3, 5 and 7, the medium was removed and 100 µL of CCK-8 was added to each well, followed by incubation for 2 h. Optical density (OD) was measured at 450 nm with a microplate reader (infinite M200 PRO). Inhibition rate was calculated as follows: inhibition rate = (OD_{450 experiment} - OD_{450 GSTP1 over-expression})/OD_{450 GSTP1 over-expression}.

Statistical analysis

Statistical analysis was done with SPSS version 13.0. Comparisons between two groups were done with t-test and data were expressed as means ± standard deviation. A value of P < 0.05 was considered statistically significant.

Results

Construction of lentivirus with GSTP1 over-expression

The mRNA of human GSTP1 was obtained from GenBank (NM_000852) and served as a tem-
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Plate for the design of primers with restriction sites. cDNA of T24 cells served as templates for PCR. The products from PCR (651 bp) were subjected to digestion with BamH I and EcoR I, followed by connection with T4 DNA ligase. The resultant products were used to infect competent cells which were then seeded into LB agar plates containing 100 μg/mL ampicillin, followed by incubation at 37°C over night. A single colony was harvested for expansion, and plasmids were extracted for digestion with restriction endonuclease BamH I and EcoR I. The products were subjected to 1% agarose gel electrophoresis (Figure 1A). The targeted plasmids were sequenced for identification. Results shown in Figure 1B indicated that GSTP1 gene was expressed in pWPXL lentivirus.

**GSTP1 significantly inhibits the proliferation of T24 cells**

T24 cells in control group and GSTP1 group were digested with 0.25% trypsin and then seeded into 96-well plates (1000 cells/well), followed by incubation at 37°C in an environment with 5% CO₂. On days 1, 3, 5 and 7, cells were treated with CCK-8, and OD was measured at 450 nm. Results showed T24 cells with GSTP1 over-expression showed significantly inhibited proliferation when compared...
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with control group, and the inhibition rate was 32.03 ± 4.68% (Figure 2; n = 3).

**GSTP1 significantly inhibits colony formation in T24 cells**

T24 cells in control group and GSTP1 group were digested with 0.25% trypsin and then seeded into 96-well plates (1000 cells/well), followed by incubation at 37°C in an environment with 5% CO2. On day 10, cells were fixed in paraformaldehyde and stained with crystal violet. Colonies could be observed macroscopically (Figure 3A). Colony counting showed the number of colonies was 463.67 ± 38.16 (n = 3) in control group and 248 ± 18.46 (n = 3) in GSTP1 group, and analysis showed the colony formation of T24 cells with over-expression of GSTP1 was markedly suppressed (P < 0.01) when compared with control group (Figure 3B).

**GSTP1 arrests T24 cells in G0/G1 phase**

Cells in logarithmic growth phase in control group and GSTP1 group were digested with trypsin and harvested by centrifugation. These cells were stained with PI and cell cycle was determined by flow cytometry (Figure 4A). Results showed the proportion of cells in G0/G1 phase, S phase and G2/M phase was 47.31 ± 0.20%, 14.76 ± 0.54% and 37.92 ± 0.34%, respectively, in control group (n = 3) and 58.43 ± 0.86%, 11.84 ± 0.49% and 29.73 ± 0.68%, respectively, in GSTP1 group (n = 3). Statistical analysis showed T24 cells with stable GSTP1 over-expression were arrested in G0/G1 phase (P < 0.01; Figure 4B).

**GSTP1 up-regulates p21 expression in T24 cells**

Total proteins were extracted from cells in control group and GSTP1 group, and 20 µg of proteins were subjected to electrophoresis for the detection of expression of GSTP1 and p21. Results showed the p21 expression in T24 cells with GSTP1 over-expression increased markedly when compared with control group (Figure 5A). The OD of p21 was 0.1806 ± 0.0655 in control group (n = 3) and 1.9867 ± 0.1456 in GSTP1 group (n = 3) (P < 0.01; Figure 5B).

**Discussion**

Increased GSTP1 expression has been found in a variety of cancers, and in vivo studies also confirm that the positive rate of GSTP1 increases gradually in normal tissues, benign lesions and cancers, and the poorer the differentiation and the lower the clinical grade, the higher the GSTP1 expression is [5]. Available studies fail to confirm that GSTP1 can prevent cancerogenesis, and the increased GSTP1 expression in cancers and precancerous lesions only suggests that GSTP1 involves in the cancerogenesis, but the specific mechanisms are still unclear. In the present study, CCK-8 assay and colony formation assay were performed in T24 cells with GSTP1 over-expression, and results showed the proliferation of T24 cells with stable GSTP1 over-expression was significantly inhibited when compared with control group (P < 0.01).

Cell proliferation and cell cycle progression are two processes with close relationship. For the end of one mitosis to the end of next mitosis, cells divide into 2 daughter cells. A cell cycle includes the preparation phases (G1, S and G2 phases) and mitotic phase. In addition, cells in
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G1 phase may be also in a quiescent state, and they do not grow and differentiate. This is also known as G0 phase. When the cell cycle progression is abnormal, cells may present inharmonious proliferation, and cells with genetic mutation are not susceptible to apoptosis, resulting in occurrence of cancers. Cell cycle progression is strictly controlled by cyclin-dependent kinases (CDKs). The positive regulation of CDK activity is done by a group of proteins known as cyclins. Crystal structure analysis of CDK monomer and CDK-cyclin complex shows the catalytic center of CDK in the form of monomers is covered and thus they have no activities. Once cyclin binds to CDK, the protein structure of CDK changes, and the catalytic center is exposed, leading to the activation of CDK [6]. CDK activity is not only positively regulated by cyclin but negatively regulated by another group of proteins known as cyclin-dependent kinase inhibitors (CKI) [7]. CKI can bind to CDK monomers or CDK-cyclin complexes to exert effects. It has been found 2 CKI families: INK4 family and Cip/Kip family. INK4 family consists of p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) and they can specifically inhibit CDK4/6. INK4 family members may bind to CDK to form stable complexes to block the binding of CDK to cyclins. Cip/Kip family includes p21 (Waf1/Cip1), p27 (Cip2) and p57 (Kip2), and they may act on CDK-cyclin complex (especially the CDK4/6-cyclinD complex in G1 phase) leading to their inactivation. In the present study, cell cycle assay and western blot assay were done in cells of control group and GSTP1 group. Results showed T24 cells with GSTP1 over-expression were arrested in G0/G1 phase and they showed significantly increased expression of p21 (P < 0.01) when compared with control group.

In the present study, our results show the proliferation of T24 cells with stable GSTP1 over-expression is markedly inhibited, and these cells are arrested in G0/G1 phase, accompanied by significant increase in p21 protein expression when compared with control group (P < 0.01). However, the specific pathways in which GSTP1 regulates p21 expression are required to be further studied.

Acknowledgements

This study was supported by National Natural Science Foundation of China (81360326); Youth Science Foundation of National Natural Science Foundation of China (81202012) and Project of Science Research and Technology Development of Guilin City (20080408).

Disclosure of conflict of interest

None.

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References

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