Increased expression of DNA repair gene XPF enhances resistance to hydroxycamptothecin in bladder cancer

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Summary

Background: Xeroderma pigmentosum group F (XPF) is an important participant in the nucleotide excision repair process. This study aimed to investigate the expression of DNA repair gene xeroderma pigmentosum group F (XPF) in bladder cancer and its clinical significance.

Material/Methods: Total RNA and protein were extracted from 45 untreated bladder cancer tissues and 21 hydroxycamptothecin (HCPT)-treated bladder cancer specimens. Real-time PCR and Western blot assay were used to detect the mRNA and protein levels of XPF, respectively. siRNA targeting XPF was used to knock down the XPF expression in T24 cells and 5637 cells, and the sensitivity of XPF-depleted cells to HCPT was measured.

Results: The XPF expressions in the HCPT-treated cancer tissues was significantly higher than those in the untreated cancer tissues at both mRNA and protein levels. Importantly, the enhanced XPF expression decreased the sensitivity of T24 cells and 5637 cells to HCPT. Furthermore, the HCPT treatment significantly increased the apoptosis of T24 cells and 5637 cells. Additionally, after the XPF gene silencing, the chemotherapeutic resistance of bladder cancer cells was significantly decreased.

Conclusions: Our results show the increased expression of XPF is involved in the chemotherapeutic resistance of bladder cancer, and decreasing XPF expression may become a promising therapeutic strategy for bladder cancer.

key words: bladder cancer • xeroderma pigmentosum group F • hydroxycamptothecin • chemotherapeutic resistance • apoptosis

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BACKGROUND

There are a variety of DNA repair mechanisms in cells that play essential roles in the prevention of gene mutations and the maintenance of genomic integrity and stability [1,2]. The enhanced expressions of DNA damage repair genes have been shown to decrease the chemotherapeutic sensitivity of cancers because most chemotherapeutic agents target the DNA damage pathways [3,4]. Among the DNA repair mechanisms, nucleotide excision repair (NER) is important and can correct the majority of DNA damage.

Generally, the NER process can be divided into 4 steps: (a) damage recognition and spliceosomal complex assembly; (b) cleavage of spiral double-stranded DNA; (c) damage removal; and (d) DNA repair, synthesis and double-strand linkage [5,6]. Many studies have shown that the expressions of NER related genes correlates with abnormal DNA repair. Xeroderma pigmentosum group F (XPF) is an important part of the NER process. XPF can heterodimerize with the excision repair cross-complementation group 1 (ERCC1) to generate the XPF-ERCC1 complex, which is responsible for the incision of damaged DNA at the 5’ end during NER [7].

Bladder cancer is one of the most common cancers, and approximately 90% are categorized as transitional cell carcinoma (TCC) [8]. The characteristics of TCC include poor chemotherapeutic sensitivity and susceptibility to relapse, both of which pose challenges for therapy. Currently, the studies on the role of NER in bladder cancer mainly focus on the recognition of DNA damage. Thus far, the expression and function of XPF in bladder cancer have not been completely clarified [8,9].

In the present study, the mRNA and protein levels of XPF in untreated and hydroxycamptothecin (HCPT)-treated bladder cancers were measured. Then, siRNA targeting XPF was introduced into T24 cells and 5637 cells, and the chemotherapeutic sensitivity of these cells was determined in the presence of HCPT. Moreover, the apoptosis of T24 cells and 5637 cells in the absence and presence of HCPT was also measured.

MATERIAL AND METHODS

Sample collection

Forty-five bladder cancer patients who were chemotherapy-naïve and 21 bladder cancer patients receiving chemotherapy with HCPT were recruited from the Southwest Hospital (Chongqing, China) and Xinqiao Hospital (Chongqing, China) from October 2008 to October 2010. There were 43 males and 23 females, with a mean age of 62.7±18.3 years. The 21 HCPT-treated patients received a second surgical intervention within 3 months to 2 years after the first surgery, during which all received HCPT treatment. All patients provided informed consent before participation in the study. The study was approved by the Ethics Committee of the Third Military Medical University.

Main reagents

Trizol reagent and reverse transcriptase M-MLV were purchased from Invitrogen (Shanghai, China). Quantitative PCR reagents (IQ™ SYBR® Green I Supermix) were purchased from Bio-Rad (CA, USA). Annexin-V-FTTC apoptosis assay kits were from Baosai Biological Technology Co. Ltd. (Beijing, China). Real-time PCR

Total RNA was extracted from 50–100 mg of bladder cancer tissues and prepared in accordance with the manufacturer’s instructions. After DNase I treatment, 2 μg of RNA was reverse transcribed with AMV reverse transcriptase into cDNA. The primers used in PCR were as follows: XPF 5'-TTTGTGAGGAAACTGTATCTGTGG-3'; 5'- GTCTGTATAGCAAGCATGTTAGG-3' (reverse); GAPDH 5'-CTCTGTGCTGCTCCTGTTCCGAC-3' (forward); 5'-TGAGGAGTGTGGCTCGGC-3' (reverse) [10]. The anticipated sizes of XPF and GAPDH were 125 bp and 69 bp, respectively.

Western blot assay

Fifty micrograms of protein from Baosai Biological Technology Co. Ltd. (Beijing, China). Total protein was quantified using the Bradford method. Approximately 100 mg of bladder cancer tissue was homogenized, and then suspended in 1 ml of pre-cooled lysis buffer for Radio-Immunoprecipitation Assay (RIPA) for 15 sec, followed by incubation on ice for 10 min. The cells were then sonicated at 100 W with 5-sec pulses. The lysates were centrifuged at 12,000 × g for 10 min at 4°C, and the supernatants were harvested. The concentrations of total protein were determined using the Bradford method.

Fifty micrograms of protein were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes at 20 V for 15 min. Membranes were blocked in 5% skim milk for 4 h and washed three times in Tris-buffered saline (TBS) for 5 min each. Subsequently, membranes were incubated at 4°C overnight with goat anti-human XPF polyclonal antibody (1:200) (Santa Cruz Biotechnology, USA) and then with HRP-conjugated rabbit anti-goat IgG (1:3000; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) at room temperature for 2 h. Membranes were treated with enhanced chemiluminescence (ECL) reagents (Pierce, USA), and bands visualized by autoradiography using X-ray film (Fujifilm, Japan). The Quantity One Imagine System and analysis software (Bio-Rad, USA) were used to analyze the bands quantitatively. β-actin was used as an internal control. The expressions of target protein were normalized by that of β-actin.
Cell culture

T24 cells and 5637 cells were stored at –70°C before use. The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with high glucose (Gibco, CA, USA) and 10% fetal bovine serum (FBS) in a 5% CO₂ incubator at 37°C. Generally, the cells were passaged every 2–3 days, and those undergoing passaging 3–5 times were used for experiments.

Plasmid construction for siRNA oligos targeting XPF in T24 and 5637 cells

An effective target sequence of XPF gene was identified (5'-GGATGAAGCCCTCAGCGAT-3') using the published sequence: http://www.ncbi.nlm.nih.gov/nuccore/NM_005236.2. As a template, the oligonucleotide chains were designed based on the base-pairing rule.

A siRNA SMARTPool designed to target human XPF (catalogue number 1299003) was purchased from Invitrogen. A non-targeting siRNA pool was used as a control.

Detection of sensitivity to chemotherapy

Cells (1×10⁶/ml) were seeded in a 96-well plate (100 µl/well), and 3 wells were used for each treatment. The cells were treated with HCPT (5 µmol/L) for 4 h. Cell viability was measured at different time points after the addition of MTT (0.12 mg/ml), which represents the sensitivity to chemotherapy. The absorbance was measured at 492 nm using a microplate reader (Bio-Rad, USA).

Detection of apoptosis

Cells were treated with 5 µmol/L HCPT for 4 h and then with 0.1% trypsin for digestion. After centrifugation at 1000 rpm for 5 min, the supernatant was removed and the cells were harvested and washed in PBS twice. Cells were resuspended and cell density was adjusted to 1×10⁶ cells/ml.

One hundred microliters of Annexin-V-FITC was added to the HCPT-treated cells followed by incubation for 10–15 min at room temperature in the dark. The cells were centrifuged at 1000 rpm for 5 min and washed in PBS twice. Apoptotic cells were detected using a FACScan Flow Cytometer (Becton Dickinson, USA), and data were analyzed with CellQuest 3.0 Software (Becton Dickinson, USA).

Statistical analysis

All data are expressed as means ± standard deviation (SD). Each experiment was repeated at least 3 times, and data represent the means from at least 3 parallel samples. SPSS version 11.0 (SPSS Inc., Chicago, USA) was used for statistical analysis. Analysis of variance (ANOVA) and Student’s t-test were used to determine the differences among and between groups, respectively. A P value of less than 0.05 was considered statistically significant.

RESULTS

XPF expression increased in HCPT treated bladder cancer

Real-time PCR and Western blot assay indicated that both the mRNA and protein levels of XPF in the HCPT treated bladder cancer tissues were significantly higher than those in the untreated bladder cancer tissues (P<0.01) (Figure 1). These results suggest that XPF is significantly up-regulated in the presence of chemotherapy. Similarly, HCPT treatment markedly increased the expression of XPF (P<0.01) (Figure 1).

siRNA targeting XPF reduced XPF expression in T24 cells and 5637 cells

To identify the role of XPF in the chemotherapeutic resistance of bladder cancers, siRNA-mediated silencing of XPF gene was performed in the bladder cancer cell lines
T24 cells and 5637 cells. Introduction of siRNA targeting XPF could significantly down-regulate the XPF expression in both cell lines, as shown by real-time PCR and Western blot assay. The mRNA expression of XPF in XPF silencing cells were significantly lower than in the control cells and non-targeting cells (P<0.01). (B) Western blot assay was used to determine the protein expression of XPF in T24 cells and 5637 cells (lane 1: control cells; lane 2: non-treated cells; lane 3: XPF silencing cells). (C) The relative expressions of XPF in the T24 cells and 5637 cells. The expressions of XPF in XPF silencing cells were significantly lower than in the control cells and non-targeting cells (P<0.01).

siRNA-mediated silencing of XPF gene increased both apoptosis and sensitivity of T24 cells and 5637 cells to HCPT

siRNA-mediated silencing of XPF gene increased the apoptosis of T24 cells and 5637 cells in the presence of HCPT. HCPT at 5 µmol/L significantly increased the chemotherapeutic sensitivity of these bladder cancer cell lines when XPF expression was efficiently silenced (P<0.01) (Figure 3).

Furthermore, as compared to the untreated cancer cells, the cells with XPF silencing displayed an increased proportion of apoptotic bladder cancer cells in the presence of HCPT. The proportion of apoptotic siRNA-treated T24 cells and 5637 cells was 3 times higher than that of untreated cells (P<0.01) (Figure 4).

siRNA-mediated silencing of XPF gene increased the expressions of caspase-3, caspase-9, caspase-8 in T24 and 5637 cells following HCPT treatment

Considering that the final pathway leading to the execution of apoptosis is the activation of a series of proteases termed caspases, and the intrinsic (Caspase-9) and extrinsic (Caspase-8) apoptotic pathways converge at the activation of Caspase-3, we detected the expressions of Caspase-3, Caspase-8 and Caspase-9 in bladder cancer cells and XPF-siRNA transfected bladder cancer cells in the presence of HCPT (5 µM). As shown in Figure 5, in XPF-siRNA transfected cells, the expressions of Caspase-3, Caspase-8 and Caspase-9 were all significantly increased in
a dose-dependent manner, as compared to the bladder cancer cells with intact XPF ($P<0.05$).

**Discussion**

A variety of proteins are involved in the NER process, including XPF, a key DNA repair endonuclease. Previous studies have shown that XPF can dimerize with ERCC1, forming a functional 5’ DNA endonuclease that can specifically incision the damaged DNA at 5’ end.

Many studies have shown that the expressions of NER-related genes are related to their DNA repair capabilities. High expressions of NER-related genes correlate with low chemosensitivity of cancers [11,12]. Fautrel et al. [13] found that the expression of xeroderma pigmentosum group C (XPC) in liver cancer tissue was significantly higher than in normal liver tissue, and that high expression of XPC correlates with the decreased chemosensitivity of cancers. Reduced expression of xeroderma pigmentosum group A (XPA) in testis carcinoma was associated with high sensitivity to cisplatin [14]. Chen et al. found a direct relationship between low XPC expression and the development of human bladder cancer [8]. It has been confirmed that XPF is highly expressed in the ovarian cancer cell line (A2780 cells), which decreased the chemotherapeutic sensitivity of A2780 cells to cisplatin. Ferry et al. [15] found that the expressions of $XPF$, $XPA$ and $XPC$ were significantly up-regulated in bronchial epithelial cells after exposure to the ubiquitous environmental pollutant Benzo[a]pyrene ($C_{20}H_{12}$), which is a 5-ring polycyclic aromatic hydrocarbon with mutagenic and highly carcinogenic capabilities. Furthermore, the expressions of these genes were shown to closely correlate with the concentration of Benzo[a]pyrene. Previous studies have shown that XPF expressions are significantly increased in the non-small cell lung cancer tissues; therefore, XPF is regarded as an important factor resulting in the decreased chemotherapeutic sensitivity to HCPT [16,17]. In the present study, our results also showed that the mRNA and protein expressions of XPF were significantly up-regulated following treatment with HCPT. Hence, we speculate that the increased XPF expression might be involved in the chemotherapeutic resistance of cancer cells to HCPT treatment.

Many studies have indicated that both the depletion of NER-related genes in cells and the knockout of NER-related genes in animals can greatly increase sensitivity to chemotherapeutic agents. Thompson et al. [18] found that XPF-deficient Chinese hamster ovary (CHO) cells were sensitive to various types of radiation, and nodularin could reduce the NER capacity in CHO cells. Furthermore, this study also suggested that nodularin could interfere with the functions of XPF and ERCC1 [19], which attenuated the activity of XPF and ERCC1, resulting in an increased sensitivity to UV radiation.

Similarly, when the $XPF$ and $ERCC1$ genes are silenced, the sensitivity of non-small cell lung cancer cell lines (H1299 cells and H1355 cells), ovarian cancer cell line (2008 cells), and breast cancer cell line (MDA-MB-231 cells) to cisplatin was...
significantly increased [20]. In addition, the combined depletion of XPF and ERCC1 in the aforementioned cell lines lowered their DNA repair ability after cisplatin treatment. Ahmad et al. [21] observed that the sensitivity of XPF-deficient fibroblasts to γ-radiation was significantly increased, and the XPF-ERCC1 complex could increase DNA repair in Saccharomyces cerevisiae. Moreover, they speculated that, as a DNA endonuclease, the XPF-ERCC1 complex was involved in not only the repair of spiral DNA damage but also the repair of double-stranded DNA. Various studies have demonstrated that increased XPF expression correlates with the low chemotherapeutic sensitivity of testicular cancers [22,23].

Conclusions

In the present study, our results showed the siRNA-mediated depletion of XPF efficiently increased the chemosensitivity of T24 cells and 5637 cells to HCPT. In addition, increased XPF expression enhanced the chemotherapeutic resistance of bladder cancer cells, while the sensitivity of bladder cancer cells to HCPT was significantly increased after the XPF gene was silenced. Importantly, these data provide a novel approach to increasing the chemotherapeutic sensitivity of bladder cancer.

The mechanisms of chemotherapeutic resistance of bladder cancer have not yet been elucidated. Further investigations are thus required to identify ways in which the chemotherapeutic sensitivity of bladder cancer may be efficiently increased and the recurrence of bladder cancer reduced.

Conflict of interests

Authors declare no conflict of interests.

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