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Transactivation of PTGS2 by PAX5 signaling potentiates cisplatin resistance in muscle-invasive bladder cancer cells

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ABSTRACT

Cisplatin (CDDP)-based systematic chemotherapy remains the mainstay of treatment for muscle-invasive bladder cancer (MIBC). However, acquired resistance to CDDP, a multifactorial process governed by an array of signals acting at different levels, is the major problem in BC treatment. Here, we report for the first time that, expression of Paired-box gene 5 (PAX5), a B-cell essential transcription factor, was significantly induced in CDDP-resistant BC tissues and in experimentally-induced CDDP-resistant BC cells. Inhibition of PAX5 expression by shRNA treatment effectively improved CDDP sensitivity in BC cells, whereas overexpression of PAX5 potentiated CDDP resistance through supporting BC cell survival. Mechanistically, using luciferase reporter and chromatin immunoprecipitation assays, we identified prostaglandin-endoperoxide synthase 2 (PTGS2, also called COX2), a potent enzyme responsible for prostanoids formation and inflammatory response, as the direct down-stream target of PAX5. PAX5 exerted its oncogenic function during the pathogenesis of CDDP resistance via stimulation of *PTGS2* transcription. These observations collectively suggest that dysregulation of PAX5/PTGS2 cascade plays a causal role in the induction of CDDP resistance and gene silencing approaches targeting this pathway may therefore provide a novel therapeutic strategy for overcoming CDDP resistance in BC.

1. Introduction

Bladder cancer (BC), the seventh most common malignancy, is newly diagnosed in 430,000 cases worldwide in 2012 [1]. Histologically, BC is categorized into non-muscle invasive BC (NMIBC, ~70%) and muscle invasive BC (MIBC, ~30%). After initial treatment by transurethral resection (TUR), more than 15% of NMIBC eventually progress to MIBC, leading to a poor prognosis [2]. Cisplatin (CDDP)-based chemotherapy currently represents the standard treatment for MIBC. However, progress in BC treatment by CDDP remains modest over the past decades. CDDP-based neoadjuvant chemotherapy is reported to offer only ~5% improvement in 5-year survival rates. Acquired resistance to CDDP remains the main obstacle for its clinical effectiveness [3]. Thus far the elucidation of the complicated mechanisms of CDDP resistance in BC is still in its infancy, and the integrated control of this pathogenesis is clearly a multi-faceted phenomenon governed by an array of signals acting at different levels.

Paired-box gene 5 (PAX5) is a B-cell essential transcription factor. Its expression is present along the whole process of B-cell maturation. Conditional Pax5 inactivation in early and late B lymphocytes results in a deadly failure of B lymphopoiesis, demonstrating an essential role of PAX5 regulating the gene expression program of the B cell lineage [4]. From a clinical standpoint, deregulation of PAX5 accounts for multiple B-cell malignancies including diffuse large B-cell lymphoma, intraocular lymphoma and lymphoplasmacytic lymphoma [5]. Notably, the biological effects of PAX5 known to date are carried out predominantly in hematopoietic system. However, additional as yet unknown peripheral actions of PAX5 cannot be ruled out. To this end, aberrant PAX5 expression has been also reported in lung cancer, gastric and breast cancer [6]. Of particular interest, PAX5 expression is frequently observed in BC tissues than normal bladder tissues, and high levels of PAX5 are closely associated with poorer prognosis in

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BC [2]. Nevertheless, the functional details, if any, of PAX5 in such nonhematopoietic systems remain unexplored.

Compelling data evidence a close link between B-cell function and drug resistance in multiple malignancies. In this context, we aim (i) to assess the expression profile of PAX5 in CDDP-resistant BC; (ii) to reveal any alterations in CDDP sensitivity upon PAX5 manipulation and (iii) to identify the down-stream target of PAX5 signaling to provide deep insights into molecular control of CDDP resistance in BC.

2. Materials and methods

2.1. Human samples

A total of 56 patients with muscle-invasive BC, who had received CDDP-based chemotherapy, were recruited from our hospital from January 2013 to August 2015. Tumor samples from transurethral resection (TUR) were divided into "Responders" (complete response, n = 29) and "Nonresponders" (stable or progressive disease, n = 27) groups according to CT and MRI scan and cystoscopy. Moreover, bladder urothelium biopsies (n = 8) were obtained during TUR of the prostate for benign prostatic hyperplasia (BPH) and were used as controls. Written informed consent was obtained from all participants, and this study, strictly conformed to the standards set by *Declaration of Helsinki* (2008), was approved by the local ethical committee.

2.2. Cell lines

Human BC cell lines T24, UMUC3 and J82 were purchased from ATCC (Rockville, MD, USA). Cells were routinely cultured in RPMI 1640 supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere. The CDDP-resistant T24/CR and UMUC3/CR sub-lines were established by exposing T24 cells to serially elevated concentrations of CDDP (up to 3 μg/mL) for over 6 months [1]. T24/CR cells were finally maintained in culture medium with 3 μg/mL CDDP. To construct the BC cells stably deprived of PAX5 or PTGS2 expression, T24/CR and J82 cells were transfected with PAX5 shRNA or PTGS2 shRNA (Santa Cruz Biotechnology, Shanghai, China), along with their corresponding negative controls, using Lipofectamine®3000 (Thermo Fisher Scientific, Shanghai, China), followed by selection with 50 ng/mL puromycin (Thermo Fisher Scientific). To stably overexpress PAX5, J82 cells were transfected with pCMV3-PAX5 (Sino Biological, Beijing, China) using Lipofectamine®3000, followed by selection with 50 μg/mL hygromycin (InvivoGen, Hong Kong, China).

2.3. Cell viability

To assess CDDP sensitivity, BC cells at the density of 1×10^4 cells/well in 24-well plates were treated with different doses of CDDP for 72 h, followed by viability measurement using Cell Viability Assay Kit (Abcam, Shanghai, China).

2.4. Colony formation

About 400 BC cells were seeded into the six-well plate. 48 h after cell culture, CDDP (2.0 μg/mL for T24/CR and 0.5 μg/mL for J82) was added to the cultures. After 3-day treatment, cells were placed into fresh media and were cultured for additional 9 days. Colonies were finally stained with 0.5% crystal blue (Sigma-Aldrich, Shanghai, China) after 10% formalin fixation.

2.5. In vivo chemosensitivity

BC cells (5×10^6 cells/mouse) were injected subcutaneously into the left back of 8-week-old female athymic nude mice. One week after cell inoculation, mice (n = 5) were injected intraperitoneally with either PBS or CDDP (3 mg/kg/dose). Tumor volume were measured using a caliper (Thermo Fisher Scientific) every 7 days.

2.6. Quantitative RT-PCR (qRT-PCR)

Total RNA was prepared using RNeasy Mini kit (Qiagen, Shanghai, China) according to the manufacturer's instructions. After routine digestion with DNase (Sigma-Aldrich), first-strand cDNA was synthesized using SuperScript III (Thermo Fisher Scientific). Subsequent quantitative PCR was performed by SYBR green intercalation on the CFX Real-Time PCR System (Bio-Rad, Shanghai, China), with *GAPDH* serving as the internal control. Primers used in this study have been reported elsewhere [2,7].

2.7. Immunoblotting and immunohistochemistry

The procedures employed for immunoblotting and immunohistochemistry assays have been described in detail in our previous work [8]. The primary antibodies used were rabbit-anti-PAX5 and rabbit-anti-PTGS2 (Thermo Fisher Scientific), rabbit-anti-β-ACTIN (Sigma-Aldrich).

2.8. Dual-luciferase reporter assay

The human *PTGS2* promoter region (Nucleotides −793 to +114; Genbank accession number: AF276953.2) was generated by PCR amplification and cloned into the pGL4 luciferase reporter vector (Promega, Beijing, China) using In-Fusion® HD Cloning Kit (Takara, Dalian, China). Site-directed mutagenesis was achieved using QuikChange II Site-Directed Mutagenesis Kit (Agilent, Beijing, China). For reporter assay, HEK 293 T cells were cotransfected with pCMV3-PAX5 or pCMV3 vector and the luciferase reporter plasmids, along with pRL-TK Renilla reporter plasmid (Promega). 24 h after transfection, cells were treated with 0.5 μg/mL CDDP for 2 h, followed by measuring the relative luciferase activity using a Dual-Luciferase Reporter Assay System (Promega).

2.9. Chromatin immunoprecipitation (ChIP)

ChIP was performed using rabbit-anti-PAX5 (Bio-Techne, Shanghai, China) as described by our previous work [8]. Input DNA and the immunoprecipitates were then subjected to qRT-PCR using 5′-ACATAGGTATACATGCGCCATGGTGGT-3′ and 5′-ATTCTCACTCATAAGTGGGAGTTGAACAAT-3'.

2.10. Statistical analysis

Data were compared using *Student's t*-test or one-way ANOVA followed by Tukey post-hoc wherever appropriate. The correlation between *PAX5* expression and *PTGS2* mRNA expression was determined using Pearson Chi-Square test. Results are presented as mean ± SEM and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Association of PAX5 upregulation and CDDP resistance

Initial qRT-PCR analyses revealed that PAX5 expression was significantly upregulated in "Nonresponders" than that in "Responders" and normal controls (Fig. 1A). To verify this observation

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