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# Immunohistochemical analysis for cell regulatory proteins in bladder carcinogenesis induced by *N*-methyl-*N*-nitrosourea–terephthalic acid

Yuan Shi [a,b], Lunbiao Cui [a,b], Guidong Dai [b], Jianfeng Chen [a,b], Ling Song [b], Xinru Wang [a,b,*]

[a] *Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 210029, China*
[b] *Institute of Toxicology, Nanjing Medical University, Nanjing 210029, China*

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**Abstract**

To explore the cell cycle regulatory mechanism in bladder carcinogenesis promoted by terephthalic acid calculi (TPA-calculi), male Wistar rats were initiated with *N*-methyl-*N*-nitrosourea (MNU) (20 mg/kg b.w. i.p.) twice a week for 4 weeks, and then given basal diet containing 5% TPA, 5% TPA plus 4% Sodium bicarbonate ($NaHCO_3$) or 1% TPA for the next 22 weeks. Major regulatory proteins in G1 cell cycle checkpoint including $p16^{INK4a}$, cyclin-dependent kinase 4 (Cdk4), cyclin $D_1$, retinoblastoma protein (pRb) were determined during various stages of urinary bladder carcinogenesis by using immunohistochemistry. In MNU–5% TPA treated group, the incidences of overexpression of Cdk4, cyclin $D_1$ and pRb in papilloma were significantly higher than these in simple hyperplasia ($p = 0.023$, $p < 0.001$ and 0.001, respectively) and in PN hyperplasia ($p = 0.042$, 0.012 and 0.002, respectively). The incidence of absent expression of $p16^{INK4a}$ in papilloma was much higher than that in simple hyperplasia ($p = 0.004$) and in PN hyperplasia ($p = 0.02$). Our results clearly reveal that the deregulation of $p16^{INK4a}$–cyclin $D_1$/Cdk4–pRb pathway is associated with bladder carcinogenesis promoted by TPA-calculi.


*Keywords:* Carcinogenesis; Cell cycle protein; *N*-methyl-*N*-nitrosourea; Promotion; Terephthalic acid; Urinary bladder calculi

## 1. Introduction

Terephthalic acid (TPA), one of the most commonly produced chemicals in the world, had been widely used for the synthesis of certain crystalline polyester resins, films, and fibers, in combination with glycols. It is a regent for alkalis in wool, and an additive in poultry feeds (Cui et al., 2004). In 2001, more than 2 million tonnes of TPA was produced in PR China and it is predicted that 7.5 million tonnes will be manufactured by 2010 (Dai et al., 2005a). As a non-genotoxic chemical, TPA induced bladder tumor in chronic feeding studies due to formation of urinary bladder calculi, which can be inhibited by concomitant ingestion of Sodium bicarbonate ($NaHCO_3$) (Wolkowski-Tyl and Chin, 1983; Heck and Tyl, 1985). Our recent study indicated that the calculi induced by TPA had a strong promoting activity on urinary bladder carcinogenesis (Cui et al., 2006). However, the mechanism of the development of bladder tumor promoted by TPA is still unclear. The two-stage bladder carcinogenesis protocol provides an excellent model for the study of chemical promoting effect from preneoplastic changes to carcinoma. This model consists of initiation with a genotoxic carcinogen, followed by promotion with a non-genotoxic carcinogen. Genotoxic carcinogens are believed to play a role in the carcinogenic process by causing mutations in protooncogenes or tumor suppressor genes. In contrast, the mechanisms of the development of tumor promoted by non-genotoxic carcinogens are much less well understood. Previous studies have demonstrated that cell proliferation was one of the definitive biological characteristics of promoters (Cohen and Ellwein, 1990; Shibata et al., 1989a). For instance, promoters of urinary bladder carcinogenesis such as sodium saccharin and butylated hydroxyanisole (BHA) were associated with enhanced DNA synthesis in the urinary bladder epithelium (Cohen et al., 1990; Shibata et al., 1989b).

Eukaryotic cell cycle progression needs well-regulated mechanisms. There were two main checkpoints in regulation of cell cycle, one of which was positioned before the cell enters S phase

* Corresponding author at: Institute of Toxicology, Nanjing Medical University, 140 Han-Zhong Road, Nanjing 210029, China. Tel.: +86 25 86862939; fax: +86 25 86527613.
*E-mail address:* xrwang@njmu.edu.cn (X. Wang).

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(G1–S checkpoint), the other was G2–M checkpoint (Vermeulen et al., 2003). Abrogation of the late G1 cell cycle checkpoint is a critical event in the development of many human neoplasms (Sherr, 1996). Analysis of tumor-derived mutations has suggested that $p16^{INK4a}$, cyclin $D_1$, cyclin-dependent kinases 4 (Cdk4), and the retinoblastoma protein (pRb) are components of this regulatory pathway. Accumulating evidences indicated that passage through the restriction point (R point) was ultimately regulated by the Rb family of proteins in G1, and inactivation of pRb function is an essential step in tumor progression (Sherr, 1994, 1996; Weinberg, 1995, 1996). R point is defined as a point of no return in G1, following which the cell is committed to enter the cell cycle. Recent study indicated that hyperphosphorylation of pRb was associated with cyclin $D_1$/Cdk4 complexes in rat urothelial proliferation induced by aristolochic acid (Chang et al., 2006). A close association of pRb overexpression with $p16^{INK4a}$ loss has been found in human bladder cancer (Chatterjee et al., 2004). Overexpression of cyclin $D_1$ was associated with cell proliferation in a rat two-stage bladder carcinogenesis (Lee et al., 1997a). Therefore, upstream $p16^{INK4a}$, downstream pRb and Cdk4 are likely to be associated with rat two-stage bladder carcinogenesis.

In this study, we try to elucidate whether $p16^{INK4a}$–cyclin $D_1$/Cdk4–pRb pathway is involved in rat bladder carcinogenesis promoted by TPA.

## 2. Materials and methods

### 2.1. Animals

A total of 190 male Wistar rats were obtained at age of 6 weeks from Shanghai Laboratory Animal Center of Chinese Academy of Sciences. They were housed 4 in a plastic cage (475 mm × 350 mm × 200 mm) with hard wood chips for bedding, and maintained on a basal diet (Xie-Tong Biotech Co. Ltd., Nanjing, China) and tap water ad libitum. The room temperature and the relative humidity were controlled at 22 ± 3 °C and 60 ± 10%, respectively. Fluorescent lighting was provided in a 12 h light/dark cycle. The health status of the rats was supervised by two researchers each day. The ethics committee of Nanjing Medical University approved the study.

### 2.2. Chemical

*N*-methyl-*N*-nitrosourea (MNU) was purchased from Sigma (USA). Terephthalic acid (TPA) was obtained from Yi Zheng Chemical Fiber Co. (China) (purity >99.9%). Sodium bicarbonate ($NaHCO_3$) was from Shanghai Hongguang Chemical Co. Ltd. (China). MNU was dissolved at 4 mg/ml in ice-cold citrate buffer adjusted to pH 6.0 just before each treatment. TPA and $NaHCO_3$ were mixed in the appropriate concentration with powdered basal diet and then the mixture was pelleted. Cdk4 rabbit polyclonal antibody, cyclin $D_1$ mouse monoclonal antibody, $p16^{INK4a}$ mouse monoclonal antibody, Rb rabbit polyclonal antibody, mouse and rabbit Streptavidin–peroxidase (SP) kit were purchased from Santa Cruz (USA).

### 2.3. Experimental design

Rats were used in this study after a week of acclimation period. The experimental design is shown in Fig. 1. Rats were randomly divided into 10 groups. Rats in groups 2–4 were treated with MNU (20 mg/kg b.w. i.p.) twice a week for 4 weeks, and then given basal diet containing 5% TPA (group 2), 5% TPA plus 4% $NaHCO_3$ (group 3) or 1% TPA (group 4) for the following 22 weeks. Group 5 was treated with MNU in the same manner as groups 2–4 and then given basal diet for 22 weeks. Rats in groups 7–9 were given i.p. injectings of citrate buffer

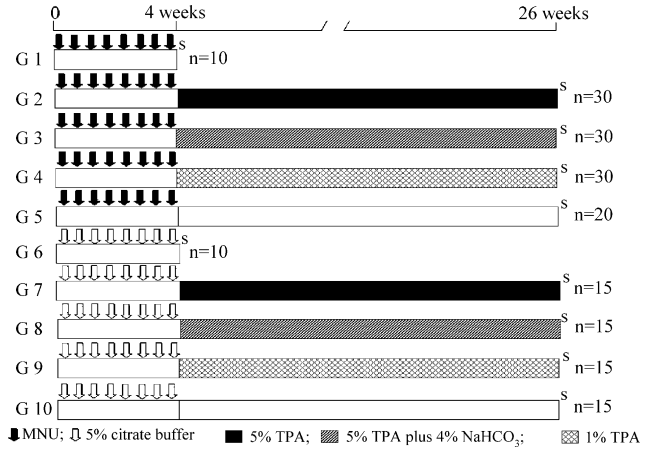


Fig. 1. Experimental design. One hundred and ninety 6-week-old male Wistar rats were divided into 10 groups; *n*, number of experimental rat; *s*, sacrifice.

(vehicle of MNU) twice a week for 4 weeks and then given basal diet containing 5% TPA (group 7), 5% TPA plus 4% $NaHCO_3$ (group 8) or 1% TPA (group 9) for the following 22 weeks. Group 10 was treated with citrate buffer in the same manner as groups 7–9 and then given basal diet for 22 weeks. All surviving rats (groups 2–5 and 7–10) were sacrificed at the end of 26 weeks. Groups 1 and 6 were treated MNU or vehicle for 4 weeks, respectively. All rats (groups 1 and 6) were sacrificed at the end of 4 weeks. The number of experimental rat in each group was presented in Fig. 1.

### 2.4. Histopathology and immunohistochemistry

After rats were euthanized by decapitation, bladder were removed and fixed in 10% phosphate-buffered formalin. Particular care was taken of the urinary tracts for stone observation. The bladder was routinely embedded in paraffin, stained with hematoxylin and eosin (H&E) and examined for histopathological assessment. Histological changes of the urinary bladder epithelium were divided into simple hyperplasia, papillary or nodular hyperplasia (PN hyperplasia), papilloma and transitional cell carcinoma (TCC), as described previously (Oyasu, 1995).

$p16^{INK4a}$, Cdk4, cyclin $D_1$ and pRb analyses of immunohistochemistry were performed using avidin–biotin–peroxidase technique as described early (Dai et al., 2005b). Briefly, after slides were deparaffinized, endogenous peroxidase activity was quenched by applying 3% hydrogen peroxide for 20 min. Antigen retrieval was performed by microwave heating. Briefly, sections were placed in 600 ml 0.01 M citrate buffer (pH 6.0) (using plastic staining racks and a vitric staining container). The sections were microwaved at high power (800 W) to boil and then at low power (200 W) for 10 min. The process was repeated once, and then the sections were allowed to cool at room temperature for further experiments. Non-specific binding was blocked by incubating sections with non-immune serum at room temperature for 20 min, and then sections were incubated overnight at 4 °C in a humidified chamber with respective primary antibodies: $p16^{INK4a}$ 1:50, Cdk4 1:100, cyclin $D_1$ 1:50 and Rb 1:100, followed by incubations with appropriate biotinylated secondary antibodies and streptavidin peroxidase. All slides were localized by a final incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with light hematoxylin.

### 2.5. Assessment of staining

Normal bladder epithelial, hyperplastic epithelial and tumorous cells whose nuclei showed antigen expression were interpreted as immunopositive, regardless of the staining intensity. At least 10 high-power fields (400×) were examined. The percentage expression of each antigen was calculated by dividing the number of immunopositive cells in all fields visualized by the total number of cells in all fields visualized, then multiplying the result by 100. More than 600 cells were counted in each side. For cyclin $D_1$, Cdk4 and pRb, ≥20% expression of positive nuclei was considered as overexpression (Dai et al., 2005b; Beasley et al., 2003). For $p16^{INK4a}$, ≤10% expression of positive nuclei was regarded as