

## Original Article

# The effects of early versus delayed castration targeting androgen on prolonging survival in a mouse model of bladder cancer

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**Abstract:** Objective: To compare the efficacy of early versus delayed surgical castration on prolonging survival and further to investigate the anticancer effect and potential value of targeting androgen in the therapeutic intervention of bladder cancer. Materials and methods: N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) was used to induce bladder cancer in male mice. Mice were randomly divided into three groups: the early castration group (on which castration was performed at 4 weeks after first time of BBN administration), the delayed castration group (on which castration was performed at 20 weeks after first time of BBN administration), and the sham-castrated group. Mice were monitored daily throughout their lifespan until cancer-related death or the progress of an obviously moribund appearance, at which time the mice were killed. Androgen receptor expression and cell proliferation and apoptosis analysis were also evaluated. Results: The average lifespan in early castration, delayed castration and sham-castrated groups were 315.8 days, 300.1 days and 254.6 days, respectively. Early castration conferred a statistically significant survival advantage when compared with the sham-castrated group ( $P < 0.05$ ). However, the difference in the lifespan between the delayed castration group and the sham-castrated group was not statistically significant ( $P = 0.198$ ). Both early and delayed castration significantly increased apoptosis of tumor cells when compared with the sham-castrated group (both  $P < 0.01$ ), which was also accompanied by a significant decrease in cells proliferation (both  $P < 0.01$ ). Prolonged survival of mice in early castration group was correlated with a lower G/B value (genitourinary tract weight/body weight) at death than the sham-castrated mice. Conclusion: Early castration had an overall survival benefit when compared with the sham-castrated treatment in BBN-induced bladder cancer mice. This finding may enhance the feasibility of androgen ablation treatment in patients with bladder cancer.

**Keywords:** Androgen receptor, bladder cancer, castration, mouse model

## Introduction

Currently, urinary bladder cancer has been recognized as one of the most common cancers with an incidence three to four times higher in males than females [1]. In 2014, there were 11170 estimated male deaths from bladder cancer and it was the fourth leading cause of cancer-related mortality among males [1]. Although the exact reason of this difference between genders is unclear, it is supposed that part of this change comes from excessive exposure to carcinogens such as industrial and environmental chemicals and cigarettes smoking in male subjects. However, even after controlling for mentioned above factors, men still have a substantially higher incidence of bladder can-

cer than women [1, 2]. Previous animal studies have shown that males are more prone to develop bladder cancer induced by certain chemical carcinogens, such as N-butyl-N-(4-hydroxybutyl) nitrosamine [BBN] than females [3, 4]. Thus, it is proposed that sex hormone status may have a role in the gender difference and contribute to the incidence and development of bladder cancer [5].

Recent progresses in bladder cancer research have demonstrated that androgen/androgen receptor (AR) signaling pathway has an important role in promoting bladder carcinogenesis as well as cancer progression. Sex steroid hormones and their receptors were evaluated and particularly AR was expressed in bladder can-

cer and most normal bladder epithelium from both male and female patients [6, 7]. The stimulatory effect of androgen on bladder cancer cell lines was also investigated [8]. Miyamoto et al. found that castrated male and wild type female mice had a lower incidence of bladder cancer induced with BBN than wild type male mice, whereas AR knockout in male or female mice did not develop any bladder cancer [4]. Johnson et al. discovered that the growth of mouse bladder cancer could be restrained from surgical castration but the suppression was decreased with dihydrotestosterone (DHT) administration [9]. Moreover, several studies showed evidence of a functional AR signaling pathway by use of antiandrogen agents and siRNA against AR in bladder cancer and a significant role for AR in bladder cancer development [4, 10, 11]. On the basis of these findings, the androgen-mediated activation of AR signaling has been considered to play an essential role in bladder carcinogenesis and cancer progression. Thus, bladder cancer may be an endocrine-related neoplasm.

Given the significance of androgen-AR signaling in the origination and progression of bladder cancer, we were curious about further exploring whether targeting the androgen has a therapeutic effect in bladder cancer. In the past few decades, the role of androgen-AR signaling in cancer development and progression was revealed using surgical castration in animal vivo studies. Surgical castration, as well as medical castration, has been the most generally used and standard treatment for locally advanced and metastatic prostate cancers for more than 60 years [12]. However, the most appropriate time to introduce androgen deprivation remains controversial [13, 14]. Arguments mainly focus on whether androgen ablation should be performed early at diagnosis or delayed until there is a disease progression with associated symptoms. In the present study, we intended to compare the efficacy of early versus delayed surgical castration on prolonging survival and further to investigate the anticancer effect and potential value of targeting androgens in the therapeutic intervention of bladder cancer.

### Materials and methods

#### *BBN-induced mouse bladder cancer model*

All animal studies were approved by Shanghai Jiao Tong University affiliated Frist People's

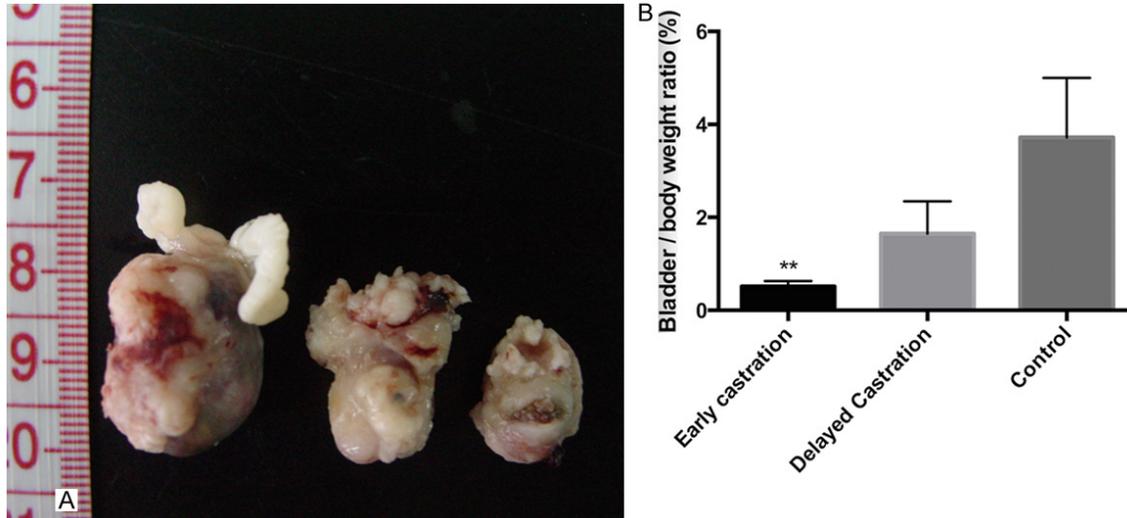
Hospital Committee on Use and Care of Animals and conducted in accordance with local humane animal care standard. Male, C57BL/6 mice (4-5 weeks old) were purchased from the Animal Centre of Chinese Academic of Science (Shanghai, China). After one week of adapting new environment, the male mice were subjected to BBN-induced bladder cancer experiments. The protocol for BBN-induced bladder cancer was used as described in a previous study [15]. Briefly, mice were given intragastric doses of BBN by using stomach tube every other day for 12 weeks. Each 7.5 mg dose was dissolved in 0.1 ml ethanol: water (20:80). The mice were weighted weekly and checked daily. Some mice died during the first two weeks in the study due to gavage errors. These mice were excluded from the final research. All mice were maintained under specific pathogen-free (SPF) conditions, allowed free access to drinking water and regular meals and kept under a controlled 12-h light/dark cycle at  $22 \pm 2^\circ\text{C}$ .

#### *Surgical castration*

Before the surgical procedure, mice were anesthetized by intraperitoneal injection of Avertin (1.25% in tertiary amyl alcohol, 250 mg/kg body weight) (Sigma, Shanghai, China). For surgical castration, mice received bilateral orchidectomy through a scrotal midline incision. Early castrations were carried out at 4 weeks, whereas delayed castrations were performed at 20 weeks after first time of intragastric administration of BBN. The sham-castrated mice were used as control. Mice were monitored daily and body weight was measured weekly.

#### *Tissue preparation*

Mice were killed when they became moribund. The criteria included huddled posture, a large palpable tumor, difficulty ambulating or an apparent moribund appearance. Mice were weighted and then sacrificed with an overdose of Avertin injection. All major organs were inspected for macroscopic evidence of primary tumors and metastases. The bladder was removed and weighted. The abdominal lymph nodes were collected and analyzed. In addition, liver, spleen, lungs, kidneys and intestines were harvested and examined for metastases. All tissues were routinely fixed overnight in 10% neutral buffered formalin, and then transferred to 70% ethanol prior to standard tissue processing. Fixed tissues were embedded in par-



**Figure 1.** Gross appearance of bladder tumors from three groups (Control, delayed castration and early castration group from left to right) (A). Column graph of G/B ratio of three groups (B). G/B ratio was calculated as (genitourinary tract weight/body weight)  $\times$  100% and analyzed with the one-way AVOVA for the different groups. Early castration (n = 4), delayed castration (n = 7), control group (n = 5). \*\*P < 0.01 (vs control group only).

affin, and sections (5  $\mu$ m thick) were mounted on slides. Sections were routinely stained with hematoxylin and eosin before histopathological examination.

#### Immunohistochemistry

Paraffin-embedded tissue sections (5  $\mu$ m thick) were deparaffinized in xylene and rehydrated in a graded ethanol series. Antigen retrieval was performed by incubating tissues with sodium citrate buffer (0.01 mol/L, pH 6.0) using a pressure cooker for 2 min at about 120°C and cooling for 30 to 60 min. Sections were washed in phosphate buffered saline (PBS) for 6 min then treated with 3% H<sub>2</sub>O<sub>2</sub> in methanol for 12 min to block endogenous peroxidases. Following washing with PBS, the sections were blocked with normal serum and then incubated with polyclonal rabbit anti-human androgen receptor (AR) (Santa Cruz Biotechnology, sc-816) at a dilution of 1:50 in PBS for 2 hours at room temperature. After washing with PBS, the slides were incubated with Envision System Anti Rabbit solution (Dako, k4003) for 30 min. Then chromogen diaminobenzidine (Vector) was applied to the samples for 3 min and then followed by a wash in PBS for 5 min. To counterstain, hematoxylin (Sigma) was applied to the specimens for 3 min, and then followed by a wash in tap H<sub>2</sub>O. The samples were then immersed in a graded ethanol series, placed in xylene, and

mounted with coverslips using mounting medium. As a negative control, PBS was used instead of the primary antibody. The stained slides were visualized under a light microscope (Olympus). Images were captured with an attached camera linked to a computer.

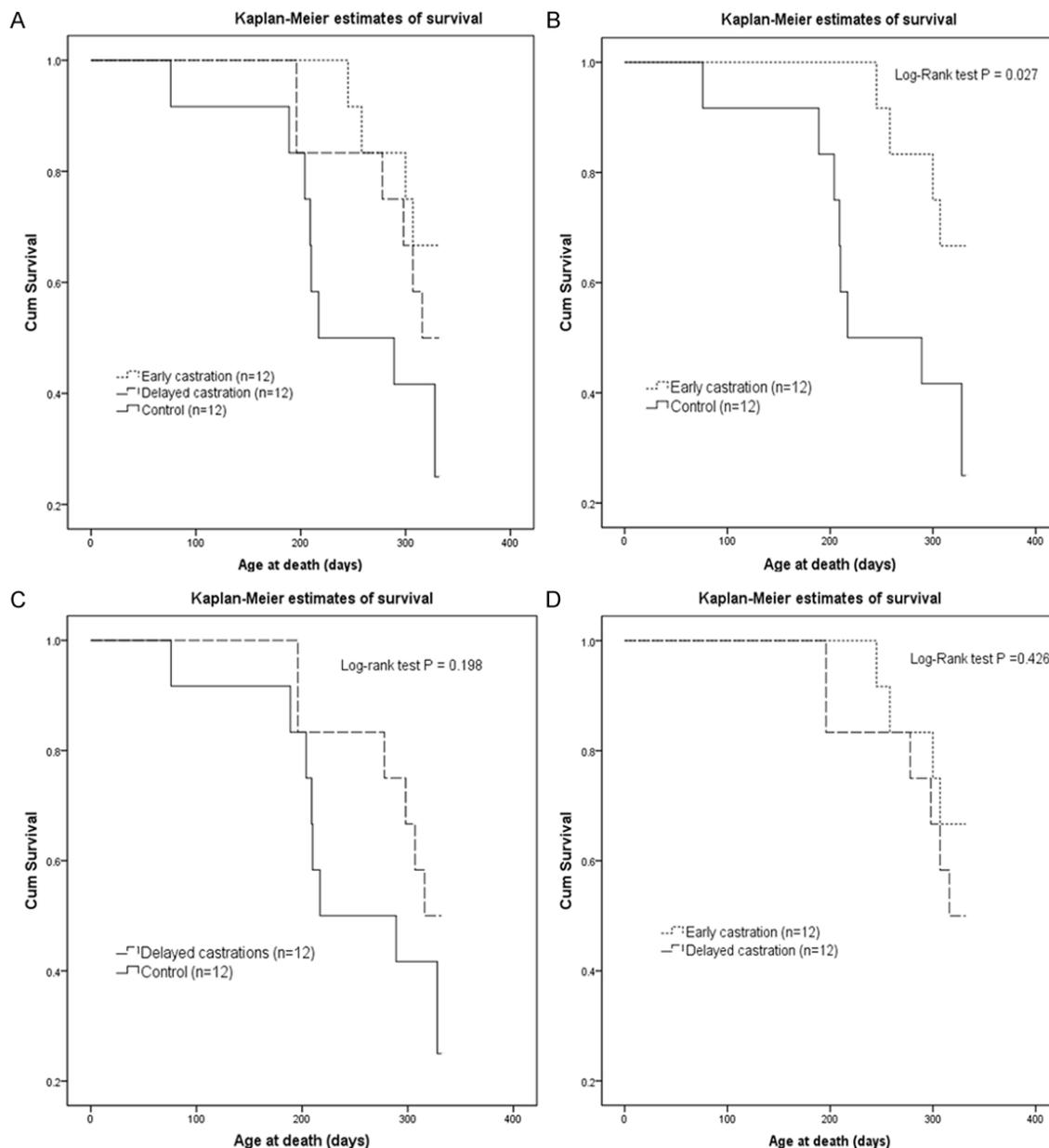
#### Cell proliferation and apoptosis analysis

The proliferative activity was detected by Bromodeoxyuridine (BrdU) incorporation, using a BrdU labeling kit (Roche Applied Science). Apoptosis was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay for in situ visualization of DNA fragmentation with commercial reagents (Roche Applied Science). The procedure was according to the manufacturer's instructions. The proliferative and apoptotic indices were measured by counting the number of BrdU-positive and TUNEL-positive cells to total cells at 400  $\times$  magnification and about 500 cells were evaluated on each specimen, in randomly selected fields. These stains were manually quantified by a single observer who was unaware of the treatment group for the tissue.

#### Statistical and survival analysis

All statistical analyses were carried out with SPSS for windows version 19.0. The data were expressed as mean  $\pm$  SE. The significance

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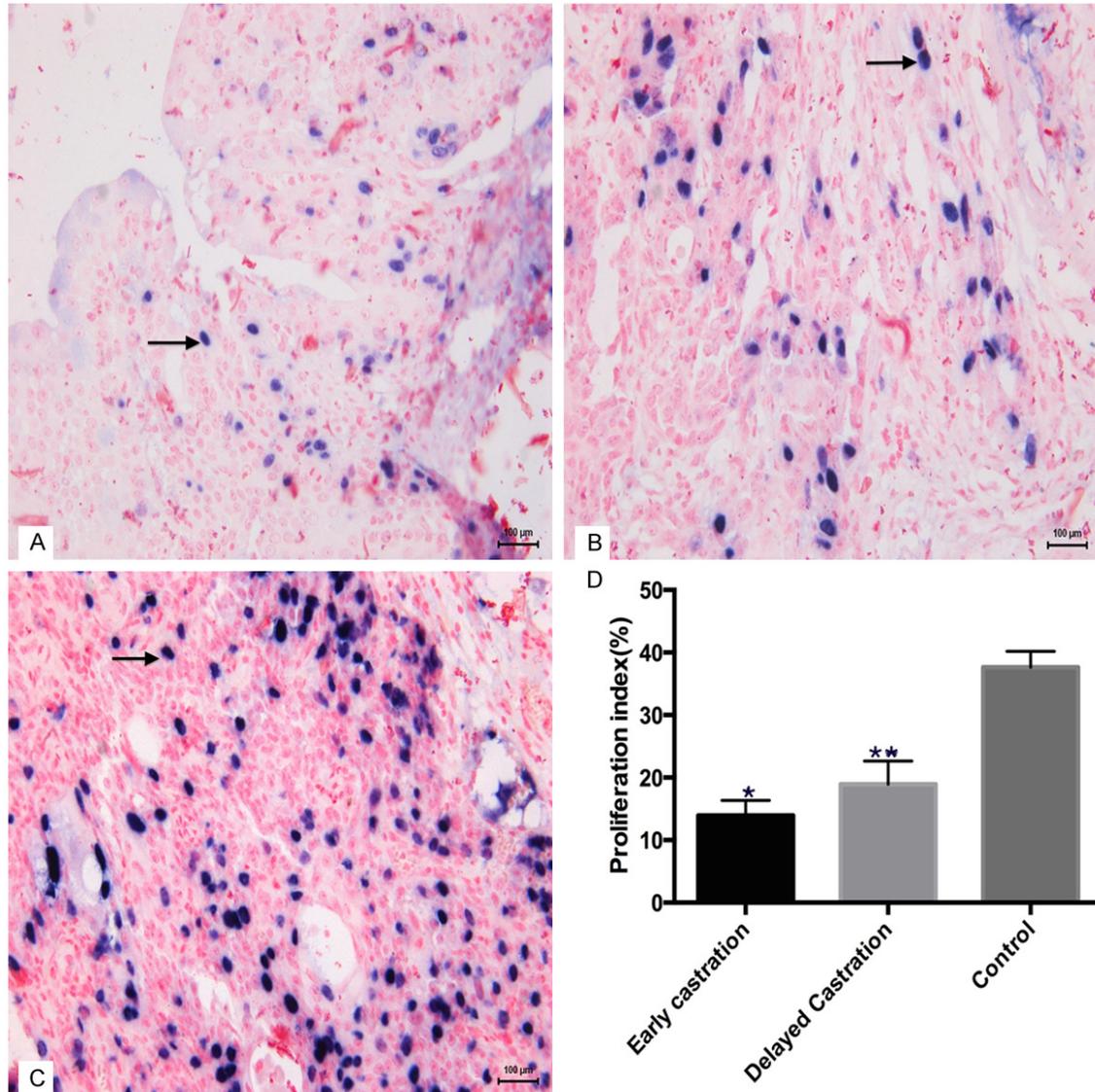
**Figure 2.** Kaplan-Meier analysis of long-term survival for early castration, delayed castration and control group in mice. A. Kaplan-Meier analysis of all three groups. B. Early castration versus control, in which the early castration group had significant longer lifespan ( $P = 0.027$ ). C. Delayed castration versus control, in which the difference between the two groups was not statistically significant ( $P = 0.198$ ). D. Early castration versus delayed castration, in which the difference between the two groups was not statistically significant ( $P = 0.426$ ).

between the control and treatment groups was performed by using the Student's  $t$  test or one-way analysis of variance (ANOVA). The Kaplan-Meier method was used to estimate survival and the differences were analyzed by the log-rank test. A value of  $P < 0.05$  was considered statistically significant in all tests. All these statistical tests were two sided.

## Results

### Bladder and body weight

At the time of necropsy, the bladder weight and body weight were determined, as a function of cancer progression. Relative genitourinary tract weight (G/B ratio), which was calculated as



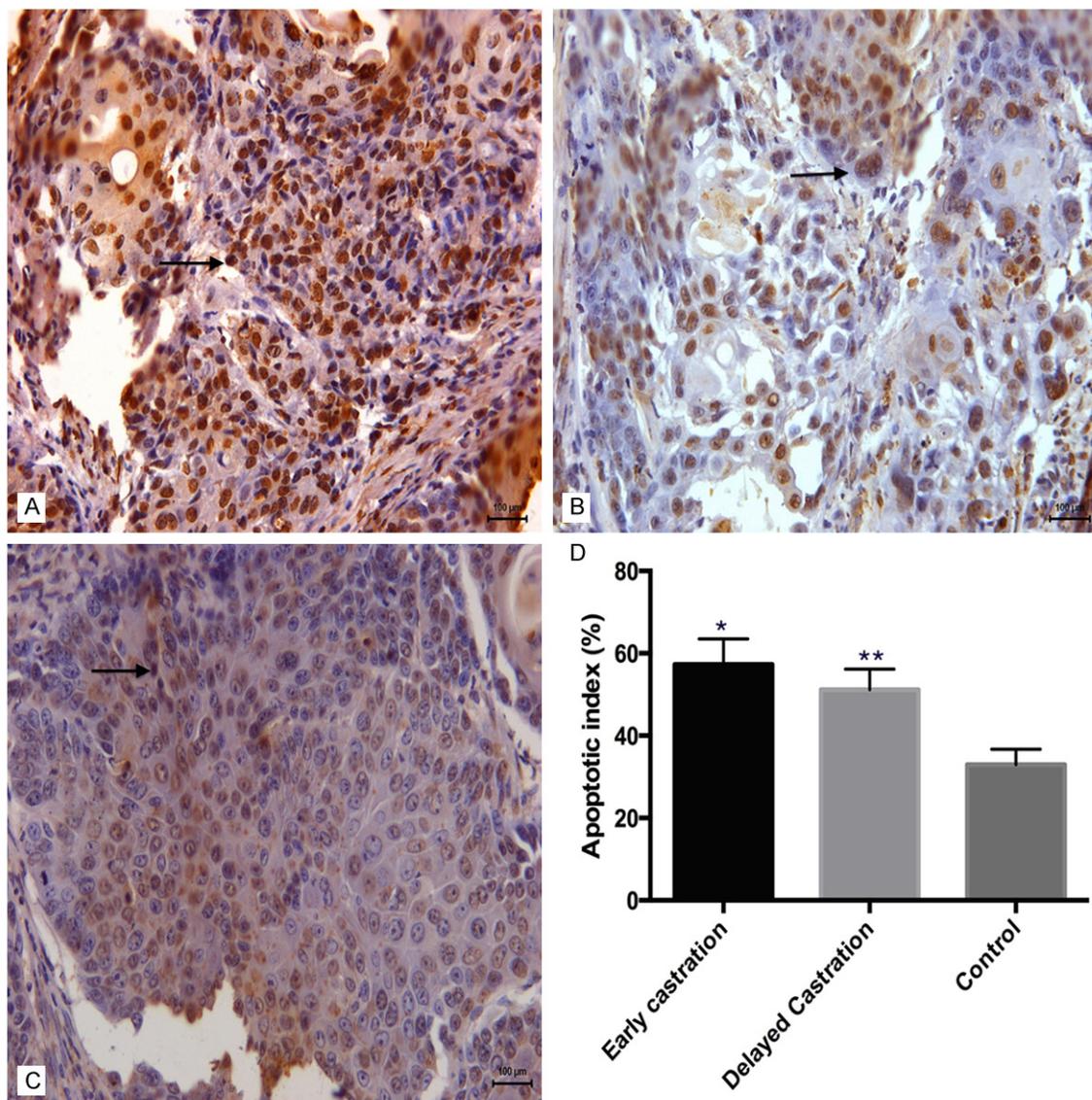
**Figure 3.** Bromodeoxyuridine (BrdU) staining of tumors for early castration, delayed castration and control groups. A representative bladder tumor from early castration group (A), delayed castration group (B) and sham-castrated group (C), with arrows exemplifying BrdU staining of proliferating cells. (D) Column graph of proliferation index of tumors from three groups. Means and 95% confidence intervals were shown. \* $P < 0.01$ , \*\* $P < 0.01$  (vs control group only).

(genitourinary tract weight/body weight)  $\times$  100%, was used to estimate the effect of castration on tumor growth in mice of bladder cancer model. The average G/B ratio of the early castration group was  $0.52\% \pm 0.11\%$ , which was significantly lower than that of the sham-castrated group, which was  $3.72\% \pm 1.28\%$  ( $P = 0.035$ ). The delayed castration group showed an average G/B ratio of  $1.69\% \pm 0.69\%$ , which was less than that of the sham-castrated group, but the difference was not statistically significant ( $P = 0.105$ ). There was no statisti-

cally significant difference in the G/B ratio between the early castration and delayed castration group ( $P = 0.388$ ). The gross appearance of bladder tumors and column graph of average B/B ratio of three groups showed in **Figure 1**.

#### Survival

Survival benefit is one of the most desirable effects of any cancer therapy regimen. In this study, we explored whether castration at differ-



**Figure 4.** TUNEL assay of tumors for early castration, delayed castration and control groups. A representative bladder tumor from early castration group (A), delayed castration group (B) and sham-castrated group (C), with arrows exemplifying TUNEL staining of apoptotic cells. (D) Column graph of apoptotic index of tumors from three groups. Means and 95% confidence intervals were shown. \* $P < 0.01$ , \*\* $P < 0.01$  (vs control group only).

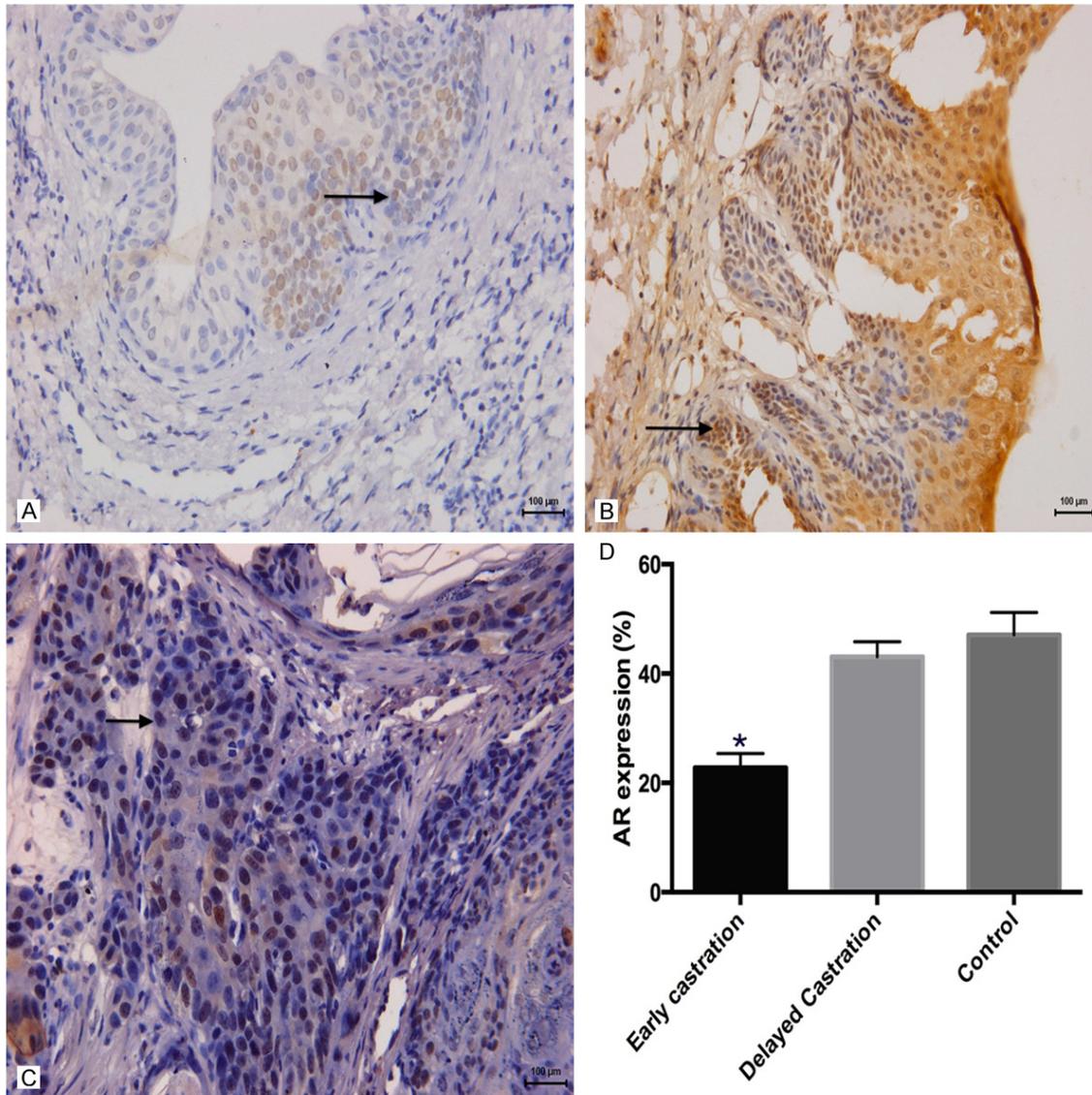
ent time points gives rise to increased lifespan in the bladder cancer model mice. The mice that were castrated at early time points got a significantly extended lifespan, with an average lifespan of 315.8 days as compared with the sham-castrated group, which had an average lifespan of 254.6 days ( $P = 0.027$ ) (Figure 2A and 2B). Mice in the delayed castration group had, on average, a 45.5-day longer and a 15.7-day shorter lifespan than those in the sham-castrated group and early castrated group, respectively, but both the differences were not

significant ( $P = 0.198$  and  $0.426$ , respectively) (Figure 2C and 2D).

#### Cell proliferation and apoptosis

Bladder cancer progression usually involves alteration of cell proliferation and apoptosis. To determine possible changes in cell proliferation and apoptosis leading to the inhibition of bladder cancer progression in castrated mice of the bladder cancer model, we did immunohistochemistry with BrdU to detect proliferative cells

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**Figure 5.** Androgen receptor (AR) staining of tumors for the early castration, delayed castration, and sham-castrated groups. A. A tumor from an early castration mouse, with an average of 22.80% positively stained cells (arrow). B. A tumor from a delayed castration mouse, with arrows pointing to a reduced number of positively stained epithelial and smooth muscle cells. C. A tumor from a sham-castrated mouse, with arrows pointing to positive staining in the majority of both the epithelial and stromal cells. D. Column graph of AR expression of tumors from three groups. Means and 95% confidence intervals were shown. \* $P < 0.01$  (vs delayed castration and control groups).

and TUNEL assay to detect apoptotic cells. Cell proliferation was similar in early castration and delayed castration group. Quantitative microscopic examination of BrdU-stained sections showed a significant decrease of BrdU-positive cells in early castration group as compared with that in sham-castrated group ( $P < 0.01$ ) (Figure 3A-D). The quantification of BrdU staining showed  $13.94\% \pm 2.40\%$  positive cells in early castration group as compared with  $37.66\% \pm 2.54\%$  positive cells in sham-castrated

group. The delayed castration group showed significant decreased number of BrdU-positive cells with  $18.93 \pm 3.72\%$  as compared to sham-castrated group ( $P < 0.01$ ) (Figure 3B and 3D). Cell proliferation index in early castration group was lower than that in delayed castration group, but the difference was not significant ( $P = 0.072$ ).

Cell apoptosis index was similar between early castration group and delayed castration group.

The number of TUNEL-positive apoptotic cells in early castration group was  $57.32\% \pm 6.18\%$  as compared with  $51.15\% \pm 4.98\%$  in the delayed castration group (**Figure 4A, 4B and 4D**). Both the early castration and delayed castration group showed statistically significant increased cell apoptosis index as compared with that in sham-castrated group with  $32.98\% \pm 3.7\%$  TUNEL-positive apoptotic cells (both  $P < 0.01$ ) (**Figure 4C and 4D**).

### *AR expression*

The AR was detected in the majority of both the smooth muscle and epithelial cells in the tumors from the sham-castrated mice (**Figure 5C**). AR staining was positive in  $22.80\% \pm 2.59\%$  of tumor cells in the early castration group as compared with  $47.10\% \pm 4.09\%$  in the sham-castrated group (**Figure 5A and 5C**). There was  $43.08\% \pm 2.76\%$  of tumor cells showing positive staining in the delayed castration group (**Figure 5B**). There was a significant decrease of AR expression in the early castration group as compared with that in the sham-castrated group and delayed castration group ( $P < 0.01$ ). AR expression was similar between delayed castration group and sham-castrated group (**Figure 5D**).

### **Discussion**

The AR, a member of the nuclear receptor superfamily, plays an important role in the development and growth of bladder cancer. The AR regulates its physiological activities by binding to androgens. The process of androgen activity was under way via an axis involving testicular synthesis of testosterone transported to target cell and bound to the AR directly or after conversion to DHT by  $5\alpha$ -reductases.

Although the bladder was not regarded as an accessory sex organ, it was derived from the urogenital sinus like other accessory sex organs such as prostate, seminal vesicles and bulbourethral gland. In the progress of human embryo, the AR could be detected in the region of the urogenital sinus and promoted the differentiation and development of sexual organs [16]. Increasing evidence from animal and human experiments has suggested that androgens conduce to urinary tract functions. In male animals studies, androgens inhibited urinary bladder detrusor muscle contraction by way of neu-

ronal regulation [17, 18]. Androgen deprivation resulted in significant alterations in the activity and expression of tissue enzymes involving cholinergic and non-cholinergic nerve actions [19, 20]. In humans, it was proposed that there is a relationship between bladder dysfunction and androgen deficiency [21]. In a study in male rats, a  $5\alpha$ -reductase inhibitor finasteride revealed minor inhibitory effects on bladder cancer development, indicating that testosterone itself might be a potent promoter of bladder carcinogenesis [22]. Molecular evidence had showed that AR pathway promote bladder tumorigenesis as well as tumor progression [23]. Several studies indicated that androgen-mediated AR signaling in urothelial cells had been supposed to be a therapeutic target in urinary bladder cancer [4, 8, 11, 24]. Testosterone, which is mostly produced by Leydig's cells in the testes, is the major active androgen that circulates within the blood of males. Higher values of circulating androgens in men may stimulate and keep higher activation state of AR signaling in carcinoma cells. Therefore, we were prone to consider that circulating androgens would be responsible for androgen-mediated AR signaling in bladder tumorigenesis and cancer development. This study was conducted with a mouse model of bladder cancer induced by BBN to study the effects of early versus delayed castration on bladder cancer progression and survival. In the experimental murine model, BBN, which can effectively induce bladder cancer from urothelial dysplasia and carcinoma in-situ to localized or invasive carcinoma, has been commonly used as an appropriate model for the experimental study of urinary bladder cancer [25]. In this study, we performed surgical castration in mice either early (at 4 weeks after first time of intragastric administration of BBN) or delayed (at 20 weeks after first time of intragastric administration of BBN or later, by which time the mice displayed palpable tumors). Intragastric method was used in order to keep dose consistency of BBN for every mouse as far as possible. Mice were monitored daily, until they died from cancer-related causes or were killed according to commonly accepted criteria.

One of the most important findings in our study was that early androgen deprivation therapy in BBN-induced bladder cancer mice had a statistically longer overall survival outcome when

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compared with delayed surgical castration, and both early and delayed treatments extended lifespan when compared with that of sham-castrated mice. However, delayed castration did not have a statistically longer overall survival advantage than that of sham-castrated mice. The most appropriate time of androgen deprivation treatment for prostate cancer is an unresolved issue. However, most of studies support the benefits of early androgen deprivation treatment in patients with aggressive prostate cancer without distant metastases. Early intervention in the treatment for cancer is very important because the development of cancer is usually complicated and multifactorial. In our study, early surgical castration in mice of BBN intragastric administration may partly prevent bladder cancer genesis. The clinical implication of our study results is that early surgical castration may actually improve the outcome of patients with bladder cancer and prolong their survival.

In this study, prolonged survival of mice in early castration groups was correlated with a lower G/B ratio at death than the sham-castrated mice. This indicates that the majority of primary tumors appear to be responsive to androgen ablation and that androgen ablation results in a regression of bladder tumor burden and extends overall survival. We observed that both early and delayed castration significantly increased apoptosis of tumor cells, which were also accompanied by a significant decrease in cells proliferation. This result was consistent with previous study [4] in which androgen deprivation therapy led to statistically significantly decreased proliferation (62%-73% reduction) and increased apoptosis (19%-81% induction) in bladder cancer cells (TCC-SUP) tumors. It is well known that the balance between proliferation and apoptosis in prostate cancer cells is regulated by androgens and the prostate cancer cells generally regress in response to androgen deprivation [26]. Our results indicate that growth of some BBN induced bladder cancers is also androgen sensitive.

In this study, we noted that AR expression was significantly lower in tumors from the early castration group than those in the delayed castration or sham-castrated groups, and the tumors in the delayed castration group revealed slightly less AR expression than those in the sham-

castrated group. Reduced expression of the AR was the normal response to castration in BBN induced bladder cancer mice. Sometimes AR was not detected in bladder tumor cells, which was possibly due to lack of AR expression. There were dissenting data as to the correlation of AR expression in bladder cancer with tumor characteristics. Most of recent studies reported that AR expression was inversely correlated with bladder tumor grade and stage [8, 27-31]. In contrast, other studies indicated that there was no significant correlation between AR expression and tumor grade and stage [32, 33]. In a large multi-institutional study, Mir et al observed no significant difference of AR expression between low-grade and high-grade in 472 patients with bladder cancer [32]. The discrepancy about the correlation of AR expression in bladder cancer with tumor characteristics may have resulted from the use of different antibodies and methodologies. However, it was worthwhile to note that AR expression level does not always accurately reflect AR activity [34].

Most of patients with bladder cancer are superficial at presentation and can be treated with a conservative manner. However, patients with superficial tumor usually take a lifelong risk of continual recurrence after complete resection of the tumor by way of a transurethral procedure, even if some of them benefit from the therapy of transurethral electroresection of bladder tumor. Thus, new approaches are required to prevent bladder tumor recurrence effectively. Androgen deprivation therapy, as a result of being widely used for patients with advanced prostate cancer, could be performed as a therapeutic approach for bladder cancer in the clinical application. In a multicenter retrospective study, Izumi et al. indicated that androgen deprivation therapy significantly reduced the risk of bladder cancer recurrence [35]. Our current findings may enhance the feasibility of androgen ablation treatment in patients with bladder cancer.

This study assessed the response to early versus delayed surgical castration and their effects on overall survival in BBN-induced bladder cancer mice. We reported that early castration had an overall survival benefit when compared with the outcome of sham-castrated mice. Further investigations are necessary to describe the precise mechanism that underlies the

effects of castration at different times on bladder cancer.

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### Disclosure of conflict of interest

None.

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