A Novel CpG Oligodeoxynucleotide Enhanced the Therapeutic Effect of Epirubicin on Bladder Tumors in Rats

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Primary research

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Abstract

Background

CpG oligodeoxynucleotides, which boast anti-inflammatory, anti-infectious, and chemotherapeutic activities, are promising immunomodulators. Recently, some preclinical studies have highlighted the potent immunostimulatory and anti-tumor effects of CpG oligodeoxynucleotides, which has aroused interest in their potential clinical effects on human cancers.

Methods

In this study, we evaluated the therapeutic effect of a new type of CpG oligodeoxynucleotide whose sequence (5'-AACGTTGTCGTCGACGTCGTCGTCAGGCCTGACGTTATCGATGGCGTTGTCGTCAACGTTGTCGTTAACGTT-3') was designed by our laboratory in combination with epirubicin in a bladder tumor rat model induced by N-methyl-N-nitrosourea instillation. Moreover, we explored the safety of the novel CpG oligodeoxynucleotide for bladder tumor therapy by observing the degree of cystolith in the bladder and comparing the results against those of Bacillus Calmette–Guérin therapy, which is a gold-standard treatment for bladder tumor.

Results

All results showed that CpG oligodeoxynucleotide combined with epirubicin significantly inhibited the growth of bladder tumors and reduced the pathological grading. As compared with bladder cells or cytokines observed under the positive control or epirubicin-alone treatment conditions, all indexes including histopathological grading, Mutation P53 gene protein expression, and Interleukin-2 (IL-2) level were significantly optimized by instillation of CpG oligodeoxynucleotide. Immunohistochemical examination indicated that CpG oligodeoxynucleotide reduced the expression of Mutation P53 gene protein in the bladder tumor rat model. Specifically, the level of IL-2 in rat serum was increased by more than 30% CpG oligodeoxynucleotide treatment combined with epirubicin. Also, in comparison with the degree of cystolith observed in the Bacillus Calmette–Guérin group, no obvious side effects were caused by CpG oligodeoxynucleotide.

Conclusions

CpG oligodeoxynucleotide as an immunomodulator can enhance the efficacy of epirubicin and presents higher safety than Bacillus Calmette–Guérin in treating bladder cancer.

1. Background

More than 12 million new cases of bladder cancer, as the 9th most common malignancy worldwide, have been reported per year[1, 2]. In 2013, bladder cancer ranked the 16^{t}place among malignant tumors in China, causing about 70,000 new cases (accounting for 2.02% of all new tumor cases) and 30,000 deaths (1.32% of all tumor deaths)[3]. Moreover, 70%–80% of bladder cancer cases are superficial, and 60%–70% of this type will recur after operation including 20%–30% that will experience an exacerbation. In clinic, transurethral resection of bladder tumors (TURBT) has been used as the primary treatment[4]. In order to improve the survival rate, patients have been given postoperative perfusions of anticancer chemicals such as gemcitabine, cisplatin, paclitaxel, docetaxel[5], and epirubicin (EPI)[6]. However, low cure rate, limited life expectancy, strong toxic side-effects and tendency to relapse were still notable problems among patients with chemotherapy. Thus, more effective options are required for bladder cancer to reduce the recurrence rate and inhibit the rapid progression of the disease into an invasive stage.

As the main treatment for non-muscle invasive bladder cancer (NMIBC), Bacillus Calmette–Guérin (BCG) immunotherapy has been used for more than 40 years. BCG stimulates cellular immune responses and reduces the risk of recurrence or progression; thus, since the 1970s, it has been recommended as a gold standard for NMIBC after TURBT[7, 8]. However, it was reported that side effects of BCG were serious, including drug-induced cystitis, hematuria, and fever[9]. Therefore, a new molecular drug with fewer side effects to replace BCG as a bladder cancer treatment has been eagerly awaited.

CpG oligodeoxynucleotides (CpG-ODNs), neotype immune stimulators and vaccine adjuvants act to enhance immunostimulatory responses[10] such as the activation of natural killer cells, T-cells, B-cells, monocytes, macrophages, and dendritic cells[11, 12]. Moreover, data have indicated that CpG-ODNs enhanced the antitumor activity of both chemotherapy and radiation therapy[13, 14] and displayed a significant effect as single agents in various mouse tumor models[15, 16]. Weigel et al. found that the gemcitabine combined with CpG-ODNs decreased the frequency of distant metastasis, inhibited tumor growth and increased the survival time among sarcoma mice[13]. Further study suggested that CpG-ODNs potentiated the antitumor efficacy of coramsine (a novel chemotherapeutic agent) in mice with malignant mesothelioma[17]. Clinical phase I or II studies proved that CpG-ODN 1826 (5’-TCCATGACGTTCCTGACGTT-3’) and CpG-ODN 7909 (5’-TCGTCGTTTTGTCGTTTTGTCGTT-3’) enhanced the antitumor activity in glioblastoma, cutaneous lymphoma, melanoma, and renal cell carcinoma by way of weekly doses as a single agent[18–20]. All these studies indicated the potential of CpG-ODNs as an antitumor drug.

In the present study, we sought to evaluate a new type CpG-ODN whose sequence was designed by our laboratory (5’-AACGTTGTCGTCGACGTCGTCGTCAGGCCTGACGTTATCGATGGCGTTGTCGTCAA

CGTTGTCGTTAACGTT-3’) to determine whether this type of CpG-ODN enhances the efficacy of EPI and has the potential to replace BCG as an immune adjuvant. We carried out histochemical and immunohistochemical assays and detected the expression of p53 andIL-2 in a bladder cancer rat model.

2. Methods

A total of 106 female Sprague–Dawley rats (weight of 200±20 g) purchased from the Guangdong Medical Laboratory Animal Center (www.gdmlac.com.cn) were used in this study. The animals were raised in the Laboratory Animal Center of the Guangdong Pharmaceutical University at a temperature of 21ºC±2ºC for a week to adapt to the Specific-pathogen-free(SPF)laboratory environment, and the whole experiment lasted for four months in the same environment. During the study, the animals were fed a standard pellet diet and water. At the end of the study, all rats were anesthetized and decapitated without suffering[21]. The study respected the limited use of animals in line with the “three-R” system (replacement, reduction and refinement) and was approved by the Animal Experiments Ethical Committee of Guangdong Pharmaceutical University (identification no. SPF2017033). All treatments were randomized but did not involve blinding. All animal experiments were established using the ARRIVE guidelines and carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and associated guidelines, the European Union Directive 2010/63/EU for animal experiments, and the National Institutes of Health guidelines for the care and use of laboratory animals (NIH publications no. 8023, revised in 1978) where appropriate.

Study design: Female rats were chosen in order to achieve better instillation via the urethra. The whole period of the experiment was divided into two parts: the tumor establishment and treatments (Figure 1).

Sample size: The rats were randomly divided into five groups: (1) control group, where 24 rats were instilled only with 0.9% normal saline (NS) in the bladder; (2) experimental groups, where 82 rats were instilled with N-methyl-N-nitrosourea (MNU; Sigma-Aldrich, St. Louis, MO, USA) in the bladder to establish the orthotopic bladder tumor model. 10 rats of the bladder tumor model were subjected to the positive control, while another 72 rats were treated with EPI (n=24), EPI+BCG (n=24), and EPI +CpG-ODN (n=24) via bladder instillation.

*Inclusion and exclusion criteri*a: One death by hyperanesthesia occurred in the 7th week was exclusion.

Randomisation: 106 female Sprague-Dawley rats, were obtained from Guangdong Medical Laboratory Animal Center (Guangzhou, China) and randomly divided into five groups: (1) control group (24 rats); (2) experimental groups: positive control (10 rats), EPI group (24 rats), EPI+BCG group (24 rats), and EPI +CpG-ODN group (24 rats) via bladder instillation.

Blinding

Data were coded prior to analysis during the whole experiment.

Outcome measures

Tissue sections embedded in paraffin blocks were cut with thicknesses of 4 μm and then stained with hematoxylin and eosin. According to the World Health Organization (WHO) criteria[28] and *Koss' Diagnostic Cytology and Its Histopathologic Bases*, pathological changes in the bladder tumors were

confirmed. The serum IL-2 was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Eiaab Science Co. Ltd., Wuhan, China).

Statistical methods

All data were analyzed by a single-factor variance analysis t test using the Statistical Package for the Social Sciences (SPSS) version 19.0 software program (IBM Corp., Armonk, NY, USA), and $p < 0.05$ was used to indicate statistical significance.

Experimental animals

106 female 6-week Sprague-Dawley rats were used with weight of 200–250g.

Experimental procedures

All of the rats in experimental groups had tumors induced by MNU in the 1st, 3rd, 5th, and 7th weeks, while the rats in the blank control groups were instilled with 0.9% NS. In the 8th week, all rats in the positive control group were sacrificed to evaluate the success of tumor establishment. In the 9th, 11th, 13th, and 15th weeks, animals were treated with EPI, EPI+BCG, EPI+CpG-ODN, or 0.9% NS as a blank control separately and sacrificed in the 10th, 12th, 14th, and 16th weeks. After all the experiments were completed, the rats were anesthetized with chloral hydrate, and then their necks were severed to death. After completion of blood specimen collection and autopsy, the rat carcass were placed in a designated place and processed by a special department.

After overnight balancing in refrigerator at 4°C, 2 mg of MNU was dissolved in 0.2ml of citric acid buffer (pH: 6.0) for instillation per individual. All of the reagent mixture was used within 40 min. The novel CpG-ODN with 72-base sequence (5'-AACGTTGTCGTCGACGTCGTCGTCAGGCCTGACGTTATCGATGGCGTTGTCGTCAACGTTGTCGTTAACGTT-3') [14, 22], which was designed by our laboratory and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

All rats were fasted for 12 hours and then intraperitoneally injected with 10% chloral hydrate for anesthesia. After general anesthesia, the urethral orifices of the rats were sterilized by 75% ethanol application twice, then the disposable epidural catheters were inserted and 0.1ml of MNU was instilled into the bladder using an injection syringe. Metal clamps were used to fix the epidural catheters into the animals to ensure that the reagents were kept in the bladders for one hour. The posture of animals was changed every 30 min. Generally, after one hour of the instillation, spontaneous micturition of the rats was observed. Rats in the experimental groups (n=82) received MNU instillation for four times (on the first day of the 1st, 3rd, 5th, and 7th weeks, respectively). Meanwhile, 24 rats (control group) were instilled with 0.9% NS (Fig. 1). All of the procedures were conducted with reference to prior research[23–27]. On the last day of the 8th week, nearly all rats in the positive control group (n=9; one death by hyperanesthesia occurred in the 7th week) were sacrificed, and the whole blood was collected from the eye sockets, followed by the performance of cystectomy to collect the whole bladders. The whole blood was clotted by

centrifuge tubes at 4°C overnight, and the serum samples were separated. The bladders were dissected by longitudinal sectioning to calculate the tumor incidence. All bladder specimens were placed in 4% paraformaldehyde to prepare for hematoxylin and eosin and immunohistochemistry examination. Additionally, on the first day of the 9th week, 72 animals among the experimental groups were instilled with EPI (n=24), EPI+BCG (n=24), or EPI+CpG-ODN (n=24), and the blank control group (n=24) was instilled with 0.9% NS (Fig. 1). On the last day of the 10th week, 18 rats from EPI group (n=6), EPI+BCG group (n=6), and EPI+CpG-ODN group (n=6) and 6 rats in the control group (n=6) were sacrificed to collect blood and bladder samples as described above. Similarly, on the first day of the 11th, 13th, and 15th weeks, remaining animals in the experimental groups were instilled with EPI, EPI+BCG, or EPI+CpG-ODN, respectively, and those in the blank control group were instilled by 0.9% NS. Then, on the last days of the 12th, 14th, and 16th weeks, these rats of the experimental groups and blank control group (n=6 each group) were sacrificed to collect samples as outlined above.

Tissue sections embedded in paraffin blocks were cut with thicknesses of 4 μm and then stained with hematoxylin and eosin. According to the World Health Organization (WHO) criteria[28] and *Koss' Diagnostic Cytology and Its Histopathologic Bases*, pathological changes in the bladder tumors were confirmed. The tumor grades were as follows: no carcinoma (grade 0), slight hyperplasia (grade 0.5), light dysplasia (grade 1), moderate hyperplasia (grade 2), high dysplasia (grade 3), papillary carcinoma (grade 4), and invasive cancer (grade 5).

Slices were deparaffinized in xylene, hydrated through a series of graded alcohol, washed in distilled water and 0.01 M phosphate-buffered saline, immersed in citrate buffer (pH: 6.0), and put in a microwave for 5 min at 60°C for antigen retrieval. Then, they were placed in methanol containing 3% H_2O_2 for 10 min to block endogenous peroxidase activity and incubated with 1% bovine serum albumin for 30 min to block nonspecific antibody-binding sites. p53 antibody (Abcam, Cambridge, UK) was diluted into a working concentration (1:500) with 0.01 M of phosphate-buffered saline (pH: 7.2), and then slices were incubated at 4°C overnight. The slices were incubated with secondary antibody for 1 h, then diaminobenzidine was used as a chromogen, and the slices were counterstained with Mayer's hematoxylin. p53 resides in the nucleus, and positive staining appears brown. The results of expression were determined by the summation of immunostaining intensity and score in the positive ratio of cells[29] as follows: (1) immunostaining intensity: no staining (grade 0), faint staining (grade 1), medium staining (grade 2), or strong staining (grade 3); (2) positive ratio of cells: ≤ 5% (grade 0), 5–25% (grade 1), 25%–50% (grade 2), 50%–75% (grade 3), or ≥ 75% (grade 4); and (3) the result of summation: grades 0–1 indicate negative (−), grades 2–3 indicate positive (+), grades 4–5 indicate medium positive (++), and grades 6–7 indicate strong positive (+++). The images were collected by BIOQUANTOSTEO 2009 (n = 5 each slice), and the intensity was calculated using the Image-Pro Plus 6.0 program as mean ± standard deviation.

The serum IL-2 was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Eiaab Science Co. Ltd., Wuhan, China) following the manufacturer's protocol. The intensity was detected as indicated above.

3. Results

3.1 Establishment of the rat bladder tumor model

A total of seven rats (77.78%) in the positive control group showed successfully induced bladder tumors (Fig. 2-a), while no tumorigenic rat was observed in the blank control group (Fig. 2-d). Thus, according to the 2016 WHO criteria, the establishment of a bladder tumor animal model in this study was a success.

3.2 Histopathological changes in the bladders

In this study, layered thickening in intestinal-like cells and the large and uneven distribution of cell nuclei were observed in bladder transitional cells in the positive control group (Fig. 2-b). The observed histopathological changes of bladders indicated invasive cancer. In contrast, the bladder cell morphology in the control group was normal. After the first installation (10th week), bladder transitional cells in the EPI+BCG group were graded as demonstrating between moderate hyperplasia and high dysplasia, with indicators such as a relatively high quantity of cells, hyperchromatic pleomorphic nuclei, and uneven arrangement and distribution suggesting a local intestinal-like presentation noted (Fig. 3-i). In the EPI+CpG-ODN group, bladder transitional cells were graded as presenting between high dysplasia and papillary carcinoma. As compared with the EPI+BCG group, the number of transitional cells increased and appeared more obviously intestinal-like, with an uneven arrangement (Fig. 3-m). All of the differences showed a higher lesion degree in the EPI+CpG-ODN group. Considering the second installation (12th week), the number of bladder transitional cells in both EPI+BCG and EPI+CpG-ODN groups was decreased dramatically, although cells in two groups still maintained an uneven arrangement with an uneven distribution of hyperchromatic nuclei. However, the intestinal-like arrangement of cells had disappeared by this point in the EPI+BCG group (Fig. 3-j and -n). After the last instillation (16th week), as compared with the positive control group, the histopathological changes of the bladder in both the EPI+BCG group and EPI+CpG-ODN group showed great differences. However, no obvious differences were observed between the EPI+CpG-ODN and EPI+BCG groups. Briefly, the grading results of bladder transitional cells were lower, down to moderate hyperplasia in the EPI+CpG-ODN group and light dysplasia or moderate hyperplasia in the EPI+BCG group (Fig. 3-p and -l). Cell numbers in both groups were greatly decreased, and cell arrangement or distribution and cell morphology appeared to have gradually normalized.

3.3 Expression of p53

In this study, as compared with the other four groups, the ratio of p53-positive cells was highest in the positive control group (grade ++), meaning that the number of bladder cancer cells was largest ($p < 0.05$) (Figs. 3-c and 4 and Table 1). As compared with after EPI treatment alone, the bladder cancer cells were decreased significantly when exposed to the combined medical treatment of EPI+BCG or EPI+CpG-ODN (Fig. 4 and Table 1). During the experiment, bladder cancer cells were significantly inhibited by EPI+CpG-ODN since the first treatment and EPI+BCG since the second treatment. The ratio of p53-positive cells in the EPI+CpG-ODN group was kept at a relatively lower and more stable level than that in the EPI+BCG treatment group but without a significant difference.

Table 1
Immunostaining intensity and score of positive cells ratio in groups

Period	Blank control	Positive control	EPI	EPI+BCG	EPI+CpG-ODN
8th week	0 (−)	5 (++)	-	-	-
10th week	0 (−)	5 (++)	2 (+)	4 (++)	3 (+)
12th week	0 (−)	5 (++)	3 (+)	3 (+)	3 (+)
14th week	0 (−)	5 (++)	3 (+)	3 (+)	2 (+)
16th week	0 (−)	5 (++)	3 (+)	2 (+)	2 (+)

3.4 Serum IL-2 level

The level of IL-2 was significantly increased by all of the three treatments and both of the combination medical treatments significantly stimulated the expression of serum IL-2 in comparison with EPI alone ($p < 0.05$ and Fig. 5). Moreover, the IL-2 level was maintained in a high and stable fashion in the EPI+BCG and EPI+CpG-ODN groups without a significant difference between the groups from the second instillation onward.

4. Discussion

CpG-ODN, which contains “GTCGTT” motifs, achieves a highly immune stimulation in human and inhibits tumor progression[15, 30, 31]. In our previous study, we designed and synthesized a 72-base CpG-ODN sequence containing “GTCGTT” motif that could inhibit the growth of T24 cells (a bladder cancer cell line) effectively[32]. The purpose of the present study was to further research the function of this CpG-ODN in a bladder cancer rat model and to explore its potential as a BCG replacement in clinical practice. MNU, a common chemical carcinogen[33], is usually used for the establishment of bladder cancer rat models[34, 35].As the rat model can specifically reflect the clinical characteristics, it is helpful in researching the instillation strategy for promoting bladder tumor onset[24]. In this study, the results of the rats with MNU instillation and 77.8% positive rate of bladder tumor onset indicated the successful establishment of a bladder cancer rat model. The expression level of p53 among malignant tumors is positively correlated with pathological grading, clinical staging, and poor prognosis[29, 36]. p53 has been shown the overexpression in at least 50% of human tumors, especially in invasive bladder cancer[13, 32, 37, 38]. Here, we found that, following treatment with EPI+CpG-ODN and EPI+BCG, the expression of p53 was significantly decreased from the 12th week and remained stable at 10%–13%, which was significantly lower than that of 20% in EPI ($p < 0.05$) (Fig. 4). These data demonstrated that both CpG-ODN and BCG can enhance the effects of EPI for instillation in bladder cancer, which is similar to our previous studies[39]. Moreover, CpG-ODN[40] and BCG[41, 42] have been proven to stimulate the immune system and result in the increase of serum IL-2 concentration. In this study, the IL-2 level was increased up to 46% in the EPI+CpG-ODN group and 42% in the EPI+BCG group, with an average level of 55%, which

is significantly higher than that of 23% in the EPI-alone group (Fig. 5). In addition, we found that both CpG-ODN and BCG can reduce the tumor volume when combined with EPI since the first instillation, and the characteristics of bladder transitional cell carcinoma gradually disappeared and even devolved back to a normal state. Similarly, the quantity of bladder transitional cells was gradually decreased, and the pathological grading reduced to moderate hyperplasia in EPI combined with CpG-ODG or BCG instillation as compared with no obvious changes observed when using EPI alone (Fig. 3). All of these results showed that CpG-ODN is similar to BCG and can kill or inhibit the proliferation of tumor cells effectively and enhance the sensitization effect of chemotherapeutics like EPI. Furthermore, we observed significantly lower side effects with CpG-ODN than BCG. First, severe calculus in the inner wall was observed in six of 24 rats (25.0%) in the EPI+BCG group (Fig. 2-g, picture 2), while only one rat had calculus (4.2%) in the EPI+CpG-ODN group (Fig. 2-h, picture 4). Second, the dose of CpG-ODN (4 μg/each rat) was much lower than that needed for BCG (0.2 mg/each rat) to obtain a similar antitumor effect. Last but not least, the effect of CpG-ODN on tumor cells was significantly rapidly faster than that seen with BCG, reflected by the p53 expression of 13% in the EPI+CpG-ODN group versus that of 25% in the EPI+BCG group since the 10th week (Fig. 4) ($p < 0.05$).

5. Conclusions

In summary, our results demonstrated that CpG-ODN could enhance the effect of chemotherapeutics and inhibit the progression of bladder tumors. Moreover, the significantly lower dose and fewer side effects attributed to CpG-ODN in comparison with BCG suggested the benefit of CpG-ODN in the clinic. However, further study is needed to optimize the dose of CpG-ODN in animal models and to develop a highly specific indicator of bladder cancer to facilitate even more effective research. Overall, CpG-ODN is a promising immune adjuvant worth future comprehensive investigation.

Abbreviations

CpG-ODN, CpG oligodeoxynucleotide

EPI, Epirubicin

MNU, N-methyl-N-nitrosourea

BCG, Bacillus Calmette Guérin

p53, Mutation P53 gene protein

IL-2, Interleukin-2

TURBT, Transurethral resection of bladder tumors

NMIBC, non-muscle invasive bladder cancer

SPF, Specific-pathogen-free

NS, normal saline

ELISA, enzyme-linked immunosorbent assay

SPSS, Statistical Package for the Social Sciences.

Declarations

Ethics approval and consent to participate

A total of 106 rats were purchased from the Guangdong Medical Laboratory Animal Center (www.gdmlac.com.cn) were used in this study. All animal experiments were established using the ARRIVE guidelines and carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and associated guidelines, the European Union Directive 2010/63/EU for animal experiments, and the National Institutes of Health guidelines for the care and use of laboratory animals (NIH publications no. 8023, revised in 1978) where appropriate. The study respected the limited use of animals in line with the “three-R” system (replacement, reduction and refinement) and was approved by the Animal Experiments Ethical Committee of Guangdong Pharmaceutical University (identification no. SPF2017033).

Consent for publication

All the authors agree to publish this article in Cancer Cell International.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing Interests

Yang Luo, Xiaoyi Fu, Bin Dong, Hongsheng Men, Shulin Zhang, Sujuan Tian, Bin Han, Fafu Zhang, Lihong Yuan and Minjie Meng declare that they have no competing interests.

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Author Contribution

YL and XF progressed experimental design, animal experiments, data collection, animal experiments, and manuscript writing. BD made project development, experimental operations guiding, data collection and analysis. HM served as scientific advisor of animal experiment. SZ did data collection and served as scientific advisor of experimental design. ST served as scientific advisor of experimental operations, data collection and analysis. BH and FZ did experiments operations. LY served as advisor of manuscript editing. MM made protocol and project development, served as scientific advisor of experimental design, and manuscript editing. All authors have read and approved the manuscript.

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Conflict of interest

Yang Luo declares that she has no conflict of interest. Xiaoyi Fu declares that she has no conflict of interest. Bin Dong declares that he has no conflict of interest. Hongsheng Men declares that he has no conflict of interest. Shulin Zhang declares that he has no conflict of interest. Sujuan Tian declares that she has no conflict of interest. Bin Han declares that he has no conflict of interest. Fafu Zhang declares that he has no conflict of interest. Lihong Yuan declares that she has no conflict of interest. Minjie Meng declares that he has no conflict of interest.

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Figures

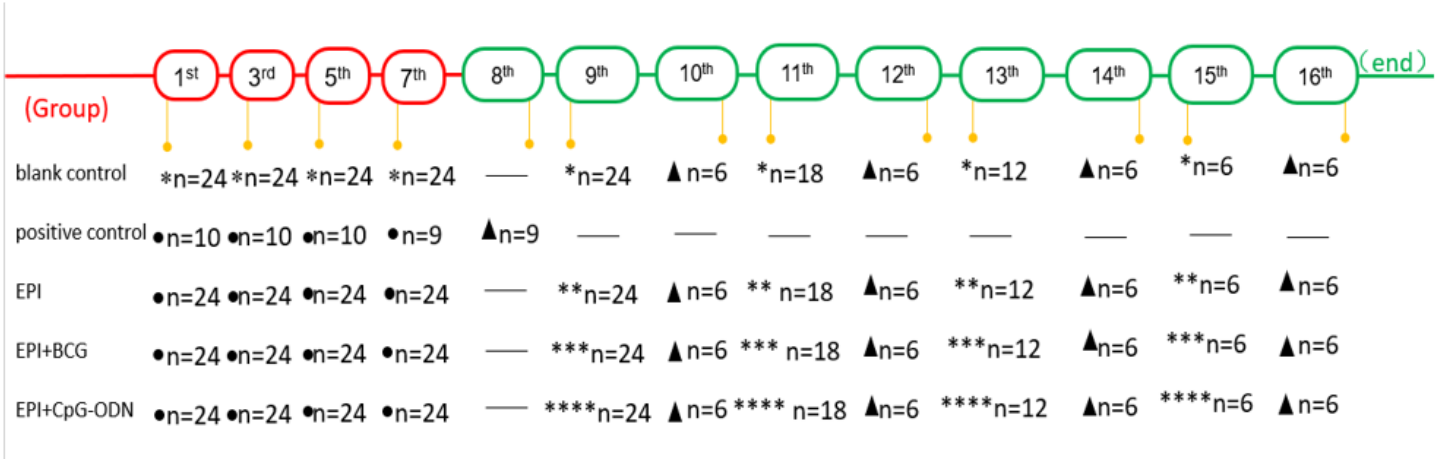


Figure 1

Summary of the animal experiment. The whole period of the experiment was divided into two parts: the tumor establishment (red) and treatments (green). All of the rats in experimental groups, including the positive control group (n = 10), EPI group (n = 24), EPI+BCG group (n = 24), and EPI+CpG-ODN group (n =

24) had tumors induced by MNU in the 1st, 3rd, 5th, and 7th weeks, while the rats in the blank control groups (n = 24) were instilled with 0.9% NS (*). In the eighth week, all rats in the positive control group were sacrificed to evaluate the success of tumor establishment. In the 9th, 11th, 13th, and 15th weeks, animals were treated with EPI, EPI+BCG, EPI+CpG-ODN, or 0.9% NS as a blank control separately and sacrificed in the 10th, 12th, 14th, and 16th weeks (n = 6 each group at each time).

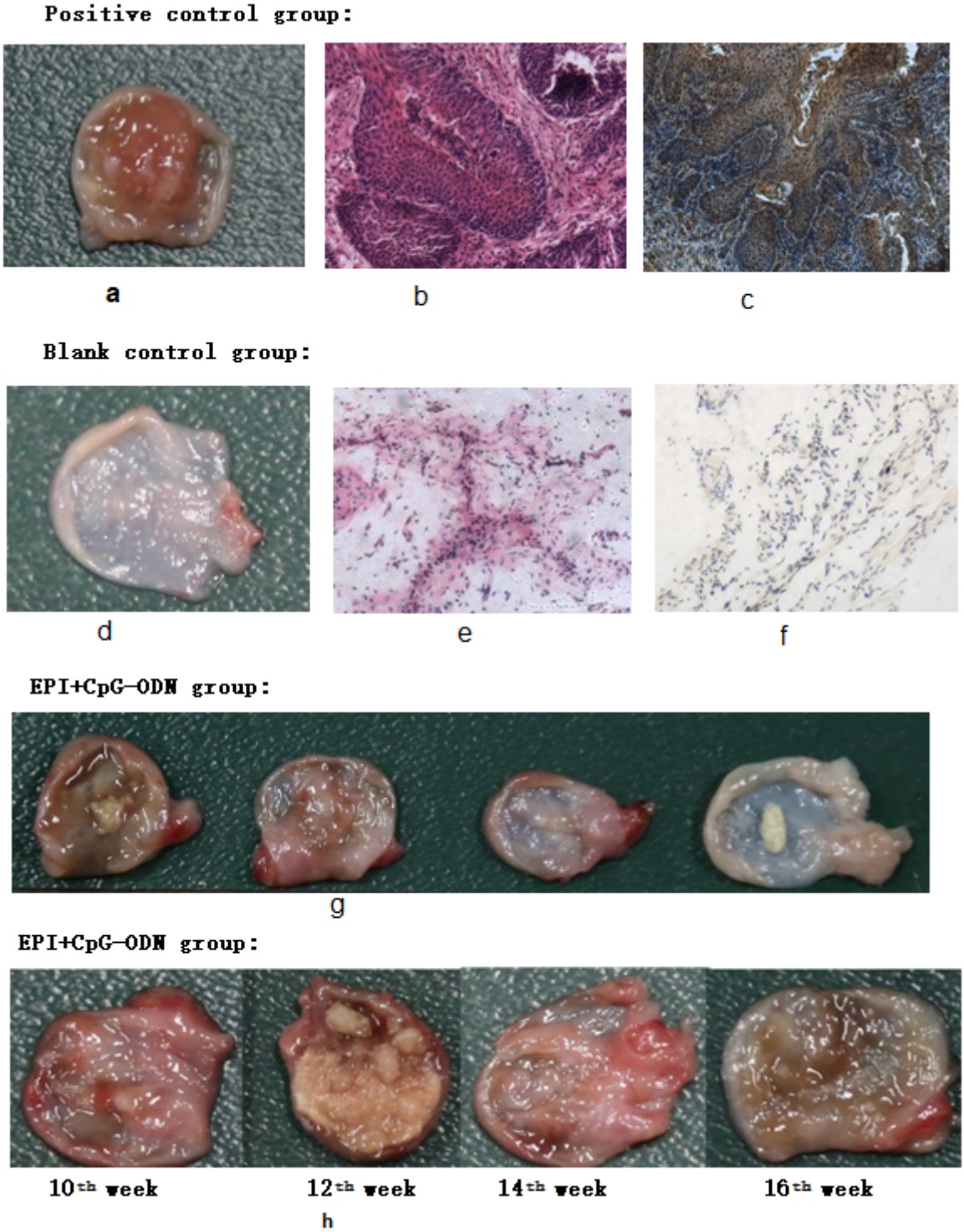


Figure 2

Establishment of a rat bladder tumor model. a–c: Positive control group. d–f: The blank control group. g: EPI+CpG-ODN. h: EPI+BCG. According to physiological characteristics, cauliflower-like tumors in reddish-brown were observed in the positive control group (a), while the bladder wall was white and smooth in the blank control group (d). According to histopathological analyses with hematoxylin and eosin, the cells in the positive control group were thicker in an intestinal-like formation, and the cell nuclei showed chromatin with uneven distribution (b, ×200), while no abnormal cells were seen in the blank control group (e, ×200). The positive p53 cells were stained with brown nuclei (c, ×200, arrow), and the negative cells showed blue nuclei (f, ×200, arrow).

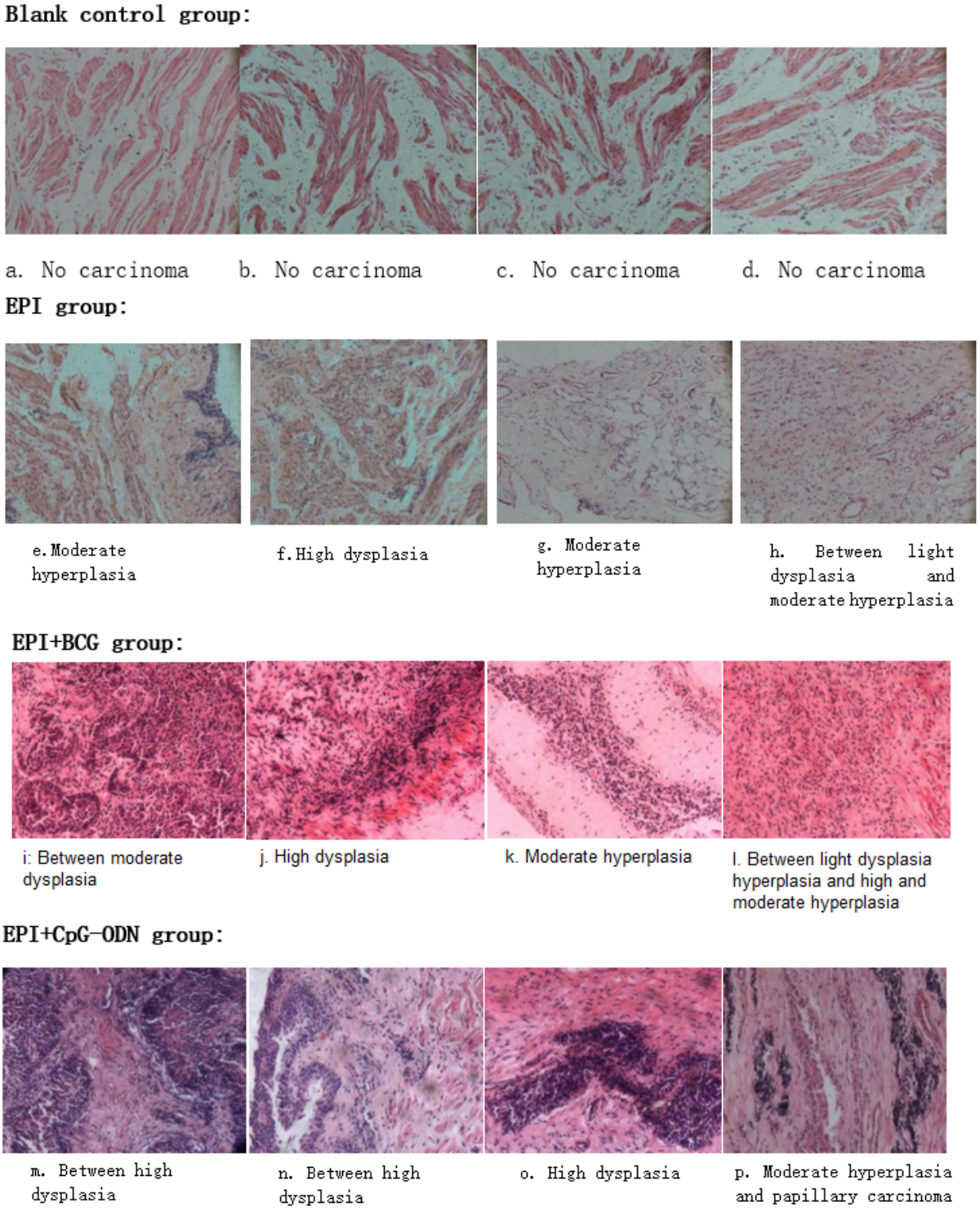
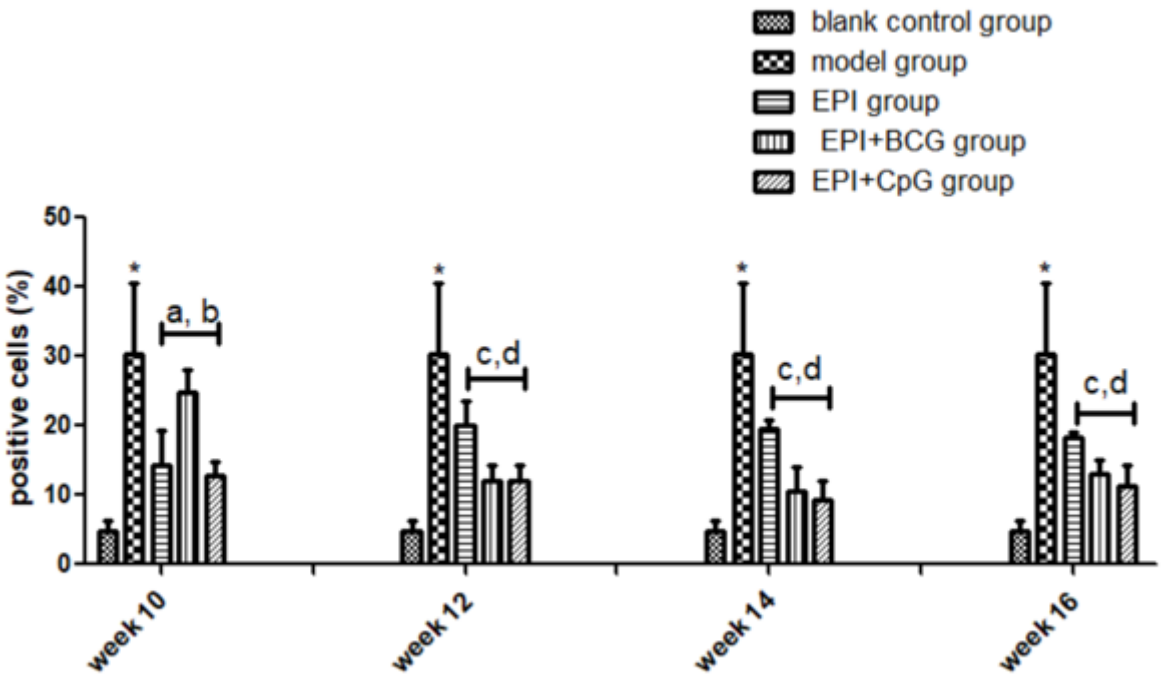


Figure 3

Histopathological changes among bladder transitional cells. a–d: Blank control group (no carcinoma; ×200). e–h: EPI group (moderate hyperplasia, high hyperplasia, moderate hyperplasia, between moderate hyperplasia and light dysplasia, respectively; ×200). i–l: EPI+BCG group (between moderate hyperplasia and high dysplasia, high dysplasia, moderate hyperplasia, between light dysplasia and moderate hyperplasia, respectively; ×200). m–p: EPI+CpG-ODN group (between high dysplasia and papillary

carcinoma, between high dysplasia and papillary carcinoma, high dysplasia, moderate hyperplasia, respectively; ×200).



A. The ratio of positive cells in each group and period

Figure 4

The ratio of p53-positive cells. *: positive control group vs. other four groups; $p < 0.05$. a: EPI group vs. EPI+ BCG group; $p < 0.05$. b: EPI group vs. EPI+CpG-ODN; $p < 0.05$. c: EPI+BCG vs. EPI+CpG-ODN group; $p < 0.05$.

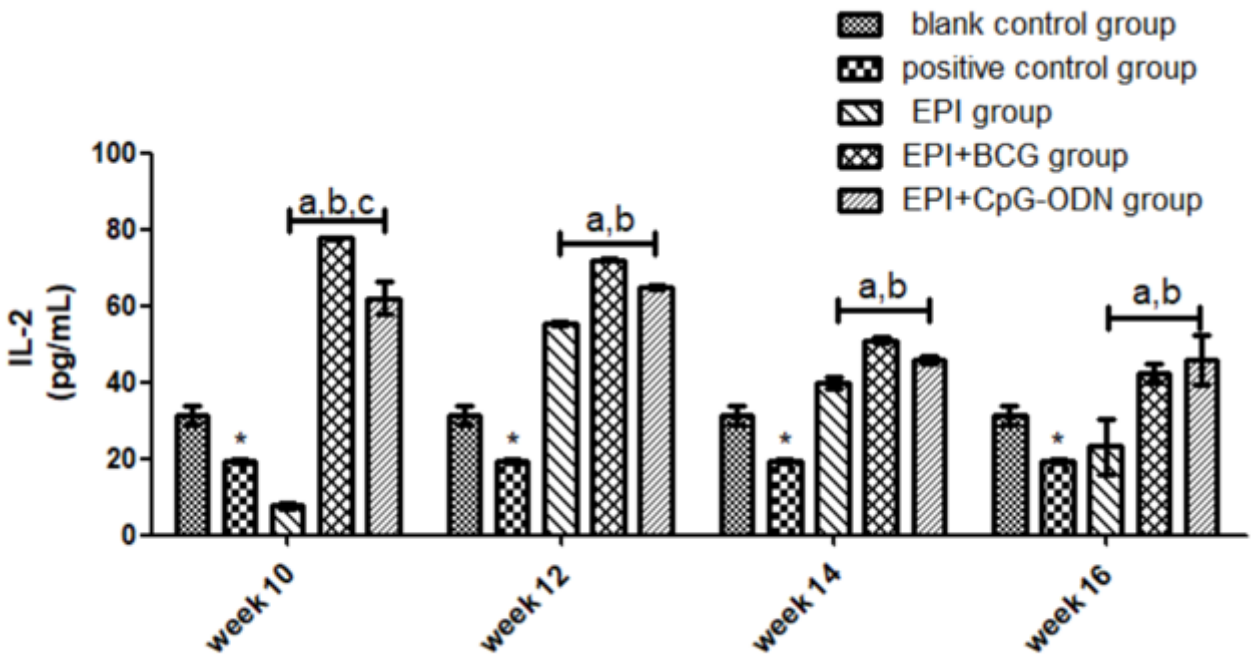


Figure 5

Changes in IL-2 level among the groups. *: positive control group vs. the other four groups, respectively; $p<0.05$. a: EPI group vs. EPI+BCG group; $p < 0.05$. b: EPI group vs. EPI+CpG-ODN group; $p < 0.05$. c: EPI+BCG group vs. EPI+CpG-ODN; $p < 0.05$.