

Implantable orthotopic bladder cancer model in Wistar rats: A pilot and feasibility study

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Abstract

Purpose: The implantable bladder cancer (BC) models allow the researchers to perform rapid and useful experiments for BC. We investigated the implantation success of BC cells obtained from Wistar rats (grown *in vitro*), into bladders of syngeneic Wistar rats, which are commonly used in the laboratories.

Methods: The Nara Bladder Tumor No.2 (NBT-II) BC cells induced with 4-hydroxybutylnitrosamine were grown with passages in Kocaeli University Center for Stem-Cell and Gene-Therapies. After urothelial denudation, 2×10^6 NBT-II cells were then implanted into bladders of 24 female Wistar rats (aged 7-8 weeks). The rats were randomly divided into four experimental groups; three instillation groups (8 per group) and one sham-operated control group consisting of 6 rats. First, second and third instillation groups were sacrificed at days 7, 14, and 21, respectively, and, bladders were histopathologically evaluated for BC according to WHO / International Society of Urological Pathology.

Results: All tumors were pT1 (including 1 rat that prematurely died at 5th day), except one rat that died prematurely at 8th day had pT2 tumor. Implantation rates were 28.58% (2/7) in the first group, and 42.85% (3/7) in the second, for a cumulative rate of 35.71% (5/14) in these two-groups (until 14th day). Interestingly, there was no tumor in the third group, but there was an inflammatory granulation tissue.

Conclusion: Seeding NBT-II cells into bladders of Wistar rats was described, successfully tested and demonstrated in this study. This implantable BC model of Wistar rats may be improved to increase the success rate of BC cell implantation in new studies with higher number of animals.

Keywords: bladder cancer model, implantable, orthotopic, syngeneic, Wistar rats

Received: 17th June 2018; Accepted: 27st October 2018; Published: 12th November 2018

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Introduction

Approximately 80% of all newly diagnosed bladder cancers (BCs) are non-muscle-invasive bladder cancer (NMIBC); carcinoma in situ (CIS), pTa, and pT1 (1). Remaining bladder tumors are higher pathological stage; \geq pT2. Transurethral resection of bladder tumor (TURBT) is the initial and standard treatment of BC. After TURBT, intravesical chemotherapeutic and immunotherapeutic agents are used to prevent the recurrence or progression of NMIBC.

The animal models of BC are roughly orthotopic (intravesical) and heterotopic (non-orthotopic [subcutaneous, etc.]). Although the subcutaneous models of BC have been frequently used in recent years (2), it should not be forgotten that these non-orthotopic models cannot represent the bladder microenvironment. On the other hand, Xiao et al. (1999) described a suitable bladder tumor model that resembles human disease. They determined the following desirable characteristics: 1. The tumor should grow orthotopically, 2. The tumor should be transitional cell carcinoma (TCC) / urothelial carcinoma origin, 3. The animal host should be immunocompetent and reasonably large, and 4. The tumor should be technically easy to develop (3).

The rodent orthotopic BC models mainly include three groups as, the chemically induced BC models, the xenograft models (implantation of human TCC into immunodeficient nude rats), and the syngeneic tumor models (implantation of carcinogen-induced BC in syngeneic immunocompetent rats) (4). Taken together, it is evident that, immunocompetent orthotopic bladder tumor models are highly desirable for BC biology and immunology. Furthermore, the studies using chemically induced bladder carcinogenesis models require several months (5-8). Hence, developing bladder tumors in a short time by implanting bladder cancer cell lines into the bladders of syngeneic immunocompetent ro-

ents might be a valuable and practical option for the research of anticancer agents.

The Wistar rats are estimated to constitute almost half of all rats being used in the laboratories today (9). However, implantation of Nara Bladder Tumor No.2 (NBT-II) cells obtained from N-butyl-N-(4-hydroxybutyl) nitrosamine-induced BC of Wistar strain, into the Wistar rats have not been clearly reported in the literature yet.

We aimed to test the possibility of transplantation of NBT-II cells into the bladders of Wistar rats, to show histopathologically the establishment of orthotopic implantable bladder tumor model, and finally to compare our data with other implantable models (Fischer 344, etc.) in this study.

Materials and methods

Ethics statement

All animal procedures were performed in accordance with the Regulation of Animal Research Ethics in Turkey on the protection of animals used for scientific purposes. The ethical approval was granted by the Kocaeli University Animal Research Ethics Committee (Project number: 3/9-2016, Kocaeli, Turkey).

Cell and Cell Culture

Rat Wistar bladder tumor (NBT-II) cells were purchased from Sigma-Aldrich and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 1% non essential amino acids (NEAA) + 1 mM NaP (sodium pyruvate) and 100 IU/ml penicillin, and 100 mg/ml streptomycin.

Animals, experimental design, and implantation protocol

Thirty adult female Wistar albino rats weighing 250 to 350 g, aged 7 to 8 weeks were used in this study. The animal studies were conducted in

accordance with Kocaeli University Experimental Medical Research Center (DETAB, Kocaeli, Turkey) regulations. The rats were randomly divided into four experimental groups; three instillation groups (8 per group) and one sham-operated control group consisting of 6 rats. All rats fed with standard diet and tap water.

Rats in instillation groups were sacrificed at 7 (first group), 14 (second group), and 21 (third group) days after inoculation. Correspondingly, in the control group, two rats were sacrificed at days 7, 14, 21 and compared with instillation groups.

After intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) injection, bladder catheterization was performed with a 20 G (Braun, Germany) angiocatheter. The rat bladders were evacuated by angiocatheter. For urothelial denudation, the bladders of instillation groups were conditioned with acid and base contents as described before (3, 10). 0.1 N Hydrochloride solution (0.4 ml) and 0.1 N potassium hydroxide (0.4 ml) were infused into the bladders and left in place for 15 seconds consequently. The bladders were then drained and washed with phosphate-buffered saline for 30 seconds. After bladder conditioning, the NBT-II cells were implanted in DMEM media by instilling the suspension (2×10^6 cells / 0.5 ml) into rat bladders rapidly. Incubation was started after the catheter was clamped. During tumor incubation, the rats were turned 90 degrees every 15 minutes to facilitate homogeneous tumor seeding in the urothelium. Finally, the catheter was removed and the rats were put into the standard cages.

Sacrification and Histopathological analysis

Two rats did not survive until the experimental endpoints. One rat from the first instillation group and one rat from the second group died at 5th and 8th day, respectively. Their bladders were collected and fixed in 4% buffered formaldehyde, after necropsy. At the end, prema-

turally dead rats' bladders were microscopically assessed together with the bladders of rats that survived till the end of the experiment.

Surviving rats were sacrificed by intraperitoneal administration of an overdose ketamine and xylazine. The lungs and livers were harvested and evaluated for macroscopic metastasis. Then rat bladders were collected and cut longitudinally from the dome to the neck. After the macroscopic assessment of the opened bladder, tissues were fixed in 4% buffered formaldehyde and subsequently embedded in paraffin for histopathological evaluation. The tissue sections were cut at 5 μ m thickness and stained with hematoxylin-eosin (H & E). Urothelial lesions were classified according to the World Health Organization / International Society of Urological Pathology Consensus (11).

Results

Initially, necropsy was performed on two rats that died prematurely. Macroscopically, no pathological findings were detected in the livers and lungs of these two rats. By contrast, mucosal irregularities were observed in their bladders and immediately they were fixed in 4% buffered formaldehyde for histopathological examination.

The bladders of each instillation group were compared with that of their control (sham-operated) counterparts. The normal urothelial tissue of sham control group is represented in Figure 1. Implantation success of the sacrificed rats in the first, second, and third group was 28.58% (2/7), 42.85% (3/7), and 0% (0/8), respectively. According to our histopathological data, the seeding of NBT-II cells achieved the establishment of implantation BC until the 14th day (35.71%, 5/14). Besides, all of these tumors were morphologically evaluated as pT1 (Figure 2A-B). Interestingly, there was no tumor in the third group, but there was only an inflammatory granulation

tissue in that group (Figure 3). All of the histopathological results are summarized in Table 1. Furthermore, masses were observed in the lungs of the two rats in the second group; These were microscopically evaluated, but they were not metastasis. They were inflammatory nod-



Fig. 1. Micrograph shows the normal urothelium from a Wistar rat in the sham-operated control group (H & E staining, magnification 20x). Str: Stroma; Ur: Urothelium

ules. Only one pT2 tumor was detected in the rat which prematurely died at 8th day. This rat is excluded from the study and its pT2 tumor is shown in Figure 4.

Discussion

To establish the rodent syngeneic implantable BC model, the BC cells (AY-27, MB49, RBT 323, etc.) were transplanted into bladders of various rats (Fischer 344, ACI, etc.) and mice (C57BL/6, etc.) after urothelial denudation (3, 4, 10, 12-15). For urothelial denudation, the mechanical damage can be achieved by electrical cauterization and epithelial abrasion as well as by chemical denudation with hydrochloride, N-methyl-N-nitrosourea, or silver nitrate. This is followed by tumor cell instillation (16). According to our recent MEDLINE® search, the establishment of an implantable BC model in Wistar rats by using self-bladder cancer cells in vivo have not been described in the literature yet. However, in a paper, the authors claimed to plant the Wistar bladders via intravesical instil-

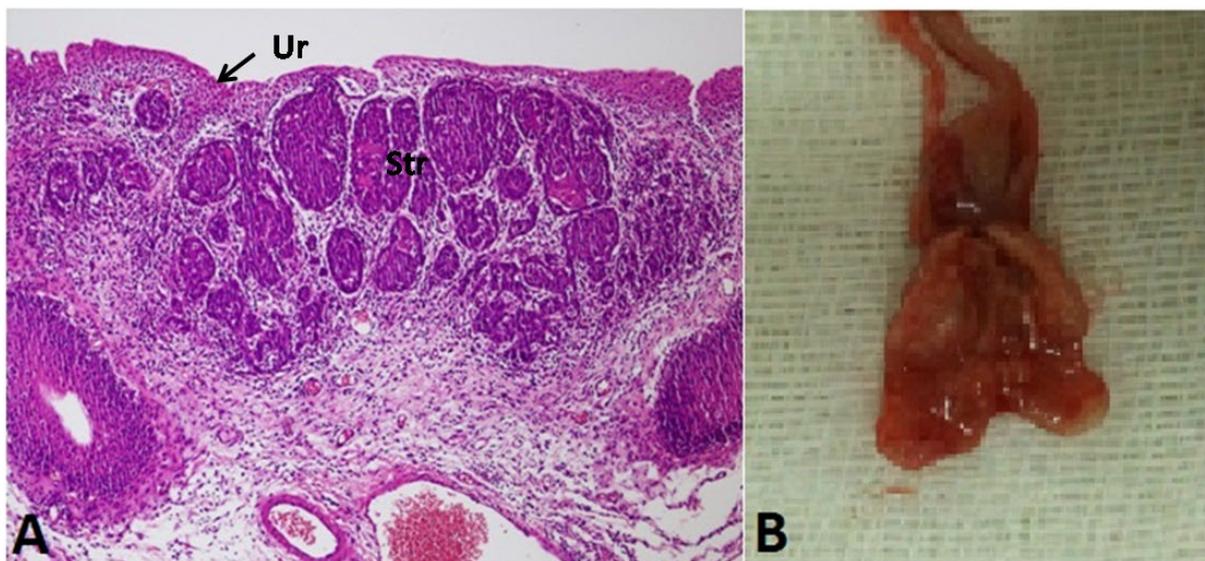


Fig. 2. The microscopic (A) image shows pT1 urothelial carcinoma in the Wistar rat bladder (H & E staining, magnification 10x). The macroscopic image (B) shows the tumor in the Wistar rat bladder. Str: Stroma; Ur: Urothelium

Table 1. Implantation ratios of the NBT-II cells in the experimental groups.

Harvesting Day	First Group (n =8)	Second Group (n = 8)	Third Group (n =8)
7 th day	2/7* (28.5%) ^α	-	-
14 th day	-	3/7* (42.8%) ^β	-
21 th day	-	-	0/8 (0%) ^ψ

* All diagnosed urothelial cancers in the first and the second groups were pT1 stage.

^α One rat from the first group died prematurely at day 5; it had a pT1 tumor.

^β One rat from the second group died prematurely at day 8; it had a pT2 tumor.

^ψ There was no tumor morphology in the bladders of the third group, but inflammatory granulation tissue were observed in their bladders.

lation (17). But poorness of histopathological data (implantation ratios, tumor stage, etc.) for the orthotopical implantation of bladder tumors in their study, diminished the reproducibility of this method in Wistar rats.

Although implantable BC models have been reported recently (3, 10, 13-15, 18), these were not in Wistar rats. The Fischer 344 rats were frequently used in BC models, especially in implantable orthotopic models. However, it is known that the Wistar rats are more commonly available in the laboratories (9). An interesting question is whether or not the tumors that were previously obtained from Wistar rats can be im-

planted in other Wistar rats, orthotopically. Xiao et al. (1999) reported that 16-17 days period is most suitable for the intravesical chemotherapy and immunotherapy studies in their implantable BC model in Fischer 344 rats. In that study, tumors were present in 62% (7/8) of the rats and all tumors (all T1 / CIS) were seen at days 12-13 (3). However, we showed 35% (5/14) bladder tumor (two rats at 7th day, three at 14th day) at the end of 14 days in Wistar rats. In addition to this, pT1 and pT2 tumors were observed in the rats that died prematurely at 5th and 8th day, respectively. Taken together, the presence of tumor in the rats that died prematurely at the 5th and 8th



Fig. 3. Microscopic image from a Wistar rat in the third group. Micrograph shows the inflammatory granulation tissue and papillary hyperplasia without any bladder cancer cell (H & E staining, magnification 100x). Str: Stroma; Ur: Urothelium; Inf. gran. tissue: Inflammatory granulation tissue.

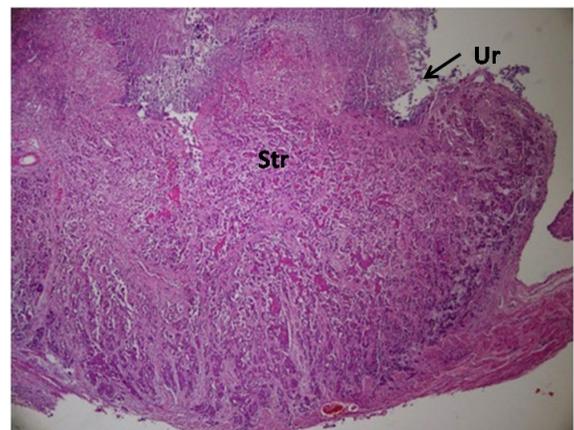


Fig. 4. The representative image of pT2 urothelial carcinoma of the Wistar rat that died prematurely at 8th day (H & E staining, magnification 10x). Str: Stroma; Ur: Urothelium

days, and in 5 of the 14 rats sacrificed in the first and second group, but the absence of tumor (but presence of inflammatory granulation tissue) in the third group suggest that the optimum waiting (implantation) period for this Wistar model is two weeks.

A solution containing two million NBT-II cells was instilled into Wistar rat bladders in the Ohana et al. (17) and our study. Ohana et al. (2004) demonstrated the development of orthotopic BC model, but the tumor stage and implantation ratio was not specified in their study. They set a 10-day experimental period and also used the rat bladders' weight to compare the normal and cancer group (17). However, in our experiment, the establishment of orthotopic BC via intravesical instillation of NBT-II cells was confirmed histopathologically and we also tested the optimal harvesting period. Moreover, we also compared this with other transplantable syngeneic rat BC models (Fischer 344, etc.).

On the other hand, Satoh et al. (2007) (10) and Xiao et al. (1999) (3) used 4×10^6 and 1 to 3×10^6 AY-27 cells in their Fischer 344 models, respectively. Using a higher concentration of NBT-II cells ($>2 \times 10^6$ cells) or any immunosuppressive agent (steroid, etc.) must be taken into consideration to increase the tumor implantation success in Wistar rats. Xiao et al. (1999) showed that tumor stage and implantation success increased with the prolonged waiting period, when 12-13 days waiting period was compared with 22-50 days in the Fischer 344 rats (3). However, in our study tumors were observed from 5th to 14th days, but we did not observe any tumor in the third group (at day 21) in Wistar rats. This paradox may be due to the fact that AY-27 cells obtained from the Fischer 344 rats are more aggressive (4) than NBT-II cells, or the immunocompetency may be more active in Wistar rats compared with the Fischer 344 rats. A prominent anti-tumoral response accompanied with inflammatory granulation tissue occurred at third week

in this model. This might be a clue to a serious tumor immunity against BC implying Wistar-related new immunotherapeutic insights. Further work is needed.

Not orthotopic but xenograft cancer models (especially subcutaneous models), with immunodeficient nude animals are highly used in new anticancer drug investigations. But, these subcutaneous models do not have appropriate bladder microenvironment for drug testing. For this reason, orthotopic and immunocompetent BC models that exhibit this microenvironment are most appropriate for intravesical immunotherapeutic and chemotherapeutic investigations. Therefore, this experimental study demonstrated rapidly grown in vivo orthotopic immunocompetent BC model for the Wistar strain. And particularly, the anti-tumoral response which became evident with the appearance of the immunocompetence effect at the third week in this model, provided clues for the clinical significance of immunotherapy in NMIBC.

In summary, we tested and histopathologically determined the optimal experimental waiting period for the seeding of NBT-II cells into bladders of Wistar rats that are commonly available and used in the experimental laboratories. The implantable Wistar rats model, described in this pilot feasibility study, may be improved to increase the success rate of BC cell implantation by new studies with higher number of animals. Then, this model can be successfully utilized in future orthotopic BC studies.

Conflict of interest

None.

Acknowledgments

The authors gratefully acknowledge the support of the Kocaeli University the Scientific and Investigational Reserve and Dr. Cemil Kayaalp

and Dr. Cafer Ali Kantar for their great assistance.

Authors' contributions

KT project development, data collection, data analysis, and manuscript writing/editing.

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MEK data collection.

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BYG data collection.

YY project development and manuscript writing.

DKY data collection, data analysis.

OD project development and manuscript writing/editing.

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