INHIBITORY EFFECTS OF COMBINATION CHEMOTHERAPEUTIC REGIMENS ON MOUSE BLADDER CARCINOGENESIS INDUCED BY N-BUTYL- N-(4-HYDROXYBUTYL)NITROSAMINE

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Abstract: Experiment 1 was conducted to examine the optimum duration of N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN) for studying promoters and inhibitors of mouse bladder carcinogenesis in mice. When the mice were treated with 0.05% BBN for 12 weeks and subsequently observed without any treatment until the 28th week, the incidence of CIS and invasive carcinoma were 20-80% and 20-60%, respectively. These results suggest that an experiment involving 12-week treatment with 0.05%BBN is useful in exploring inhibitors. Using this model, the effects of three regimens of combination chemotherapy (CAP, M-VAC, and MEC regimens) in suppressing the development of bladder carcinoma were examined in Experiments 2 and 3. The animals treated with CAP or MEC had significantly lower incidences of invasive carcinoma than BBN alone groups. As for M-VAC treated group, the incidence of superficial bladder carcinoma was significantly lower than BBN alone group. Thus each of the three combination chemotherapy regimens suppressed the development of mouse bladder carcinomas. These results suggest that CAP, M-VAC, and MEC regimens are useful in adjuvant chemotherapy for invasive bladder carcinoma. (*J Toxicol Pathol* 9: 43~49, 1996)

Key words: Bladder carcinoma, N-butyl-N-(4-hydroxybutyl)nitrosamine, Combination chemotherapy, Mouse

Introduction

Human bladder carcinoma can be mainly divided into two types: papillary non-invasive type and non-papillary invasive type. The former is often treated using transurethral resection (TUR), however this type has a high recurrence rate during follow-up. On the other hand, the latter is often fatal. These two different types of carcinoma of the urinary bladder have different patterns of growth and progression. Establishment of animal models of bladder carcinoma is essential in analyzing factors related to the onset and progression of bladder carcinoma and in obtaining basic data for useful reference when diagnosing and treating this cancer. Experimental models of human bladder carcinoma

should have a manner of histogenesis and progression similar to the natural history of human bladder carcinoma.

In our previous experiments using rats with bladder carcinoma induced by N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN), we examined the effects of treatment with anti-cancer agents on the development of BBN-induced carcinoma, and collected basic data which would be useful in preventing the recurrence of superficial bladder carcinoma in clinical cases¹⁻⁴. A similar examination was also carried out using combination chemotherapy⁵. However, since BBN-induced bladder carcinoma in rats is a model of superficial bladder carcinoma in humans, it was inappropriate to use this rat model for assessing the effects of combination chemotherapy for invasive bladder carcinoma.

In the present study, therefore, we used a mouse model of bladder carcinoma to examine the effects of three combination chemotherapy regimens; 1) CAP regimen containing cyclophosphamide (CPM), pirarubicin (THP-ADM), and cisplatin (CDDP), 2) M-

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VAC regimen containing methotrexate (MTX), vinblastine sulfate (VLB), THP-ADM, and CDDP, and 3) MEC regimen containing MTX, epirubicin hydrochloride (Epi-ADM) and CDDP.

Materials and Methods

Experiment 1

Two hundred and thirty-five 6-week-old female C3H/He mice (Oriental Bioservice, Inc., Kyoto, Japan) were used. Five animals were allocated to each plastic cage. The animals were raised in an air-conditioned room with a temperature of 22°C. They were allowed free access to water containing 0.05% BBN (Iwai Chemical, Tokyo, Japan).

All animals were divided into six groups (Fig. 1). Group 1 was composed of 50 mice, which were subdivided at random into 7 groups after 4-week BBN treatment and whose bladders were examined histopathologically every 4 weeks from the beginning of the experiment. Group 2 was composed of 50 mice, which were subdivided at random into 6 groups after 8-week BBN treatment and whose bladders were examined at intervals of 4 weeks. Group 3 comprised of 35 mice which were subdivided at random into 5 groups after 12-week BBN treatment and whose bladders were examined at intervals of 4 weeks. Group 4 was composed of 30 mice, which were subdivided at random into 4 groups after 16-week BBN treatment and whose bladders were examined at intervals of 4 weeks. Group 5 comprised 35 mice, which

were subdivided at random into 3 groups after 20-week BBN treatment and whose bladders were observed at intervals of 4 weeks. In Groups 1 through 5, no treatment was undertaken during the observation period. Group 6 was composed of 35 untreated control mice, which were divided at random into 7 groups and examined at intervals of 4 weeks until the 28th week. All animals from each group were sacrificed by an overdose of ether, and the bladders were inflated *in situ* with 10% phosphate-buffered formalin solution. After an overnight fixation, each bladder was cut vertically into 8 to 12 sections, embedded in paraffin, and stained with hematoxylin-eosin.

Experiment 2 (CAP experiment)

One hundred and five female C3H/He mice were divided into five groups (Fig. 2). The animals allocated to Groups 1 and 2 were given water containing 0.05% BBN for 12 weeks. The remaining three groups were untreated controls. The animals allocated to Groups 2 and 3 received three cycles of CAP therapy, i.e., intraperitoneal injection of physiological saline, containing CPM (45 mg/kg, Shionogi & Co., Ltd., Osaka, Japan), THP-ADM (1.5 mg/kg, Meiji Seika Kaisha, Ltd., Tokyo, Japan) and CDDP (2.0 mg/kg, Nippon Kayaku Co., Ltd., Tokyo, Japan), at intervals of 3 weeks from the 15th week on (Table 1). In Group 4, drug-free physiological saline was intraperitoneally injected in a similar way. Group 5 received no injection. All mice were sacrificed 24 weeks after the beginning of the experiment and the

Fig. 1. Experiment design.

Fig. 2. Experiment design (CAP experiment).

Table 1. CAP Regimen

	day	1	2
CPM (45 mg/kg)		↓	
THP-ADM (1.5 mg/kg)		↓	
CDDP (2.0 mg/kg)			↓
(21-day cycle)			
CPM: Cyclophosphamide,			
THP-ADM: pirarubicin, CDDP: Cisplatin			

bladders were examined histopathologically.

Experiment 3 (M-VAC, MEC experiment)

One hundred and ninety-one 6-week-old female C3H/He mice were divided into nine groups (Fig. 3). The animals allocated to Groups 1 through 4 were given water containing 0.05% BBN for 12 weeks. The remaining five groups were untreated controls. The animals allocated to Groups 2 and 5 received three cycles of M-VAC therapy, i.e., intraperitoneal injection of physiological saline, containing MTX (10

mg/kg, Lederle Japan Co., Ltd., Tokyo), VLB (0.3 mg/kg, Eli Lilly Japan KK, Tokyo, Japan), THP-ADM (1.5 mg/kg) and CDDP (2.0 mg/kg), at intervals of 4 weeks from the 12th week (Table 2). Groups 3 and 6 were the low dose MEC groups; they received three cycles of low-dose MEC therapy, i.e., intraperitoneal injection of physiological saline, containing MTX (10 mg/kg), Epi-ADM (4.0 mg/kg, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan), and CDDP (3.0 mg/kg), at intervals of 4 weeks, starting immediately after the 12-week BBN treatment. Groups 4 and 7 were high dose MEC groups; they received three cycles of intraperitoneal injection of physiological saline, containing MTX (12 mg/kg), Epi-ADM (5.0 mg/kg) and CDDP (3.5 mg/kg) (Table 3). In Group 8, drug-free physiological saline was intraperitoneally injected in a similar way. Group 9 received no injection. All mice were sacrificed in the 24th week, and the bladders were examined histopathologically.

Fig. 3. Experiment design (M-VAC, MEC experiment).

Table 2. M-VAC Regimen

	day	1	2	15	22
MTX (10 mg/kg)		↓		↓	↓
VLB (0.3 mg/kg)			↓	↓	↓
THP-ADM (1.5 mg/kg)			↓		
CDDP (2.0 mg/kg)			↓		

(28-day cycle)

MTX: Methotrexate, VLB: Vinblastine, THP-ADM: pirarubicin, CDDP: Cisplatin

Table 3. MEC Regimen

	day	1	2	22
MTX (0 mg/kg)		↓		↓
(12 mg/kg)		↓		↓
Epi-ADM (4.0 mg/kg)		↓		
(4.8 g/kg)		↓		
CDDP (3.0 mg/kg)		↓	↓	
(3.6 mg/kg)		↓	↓	

(28-day cycle), ↓: Low dose, ↓: High dose, MTX: Methotrexate, Epi-ADM: Epi-adriamycin, CDDP: Cisplatin

Results

Experiment 1

Eleven animals died of weakness for a term of experiment. They were treated with 0.05% BBN for more than 8 week, and observed more than 20 weeks. In all groups 1 through 6, histopathological examination of the bladders yielded the following results (Table 4).

Dysplasia developed in 10–20% of the animals in Group 1, in which they were treated with 0.05% BBN for 4 weeks and subsequently observed without BBN treatment until the 28th week at intervals of 4 weeks.

In Group 2 where BBN was given for 8 weeks and observation was subsequently made at intervals of 4 weeks until the 28th week, the incidence of dysplasia was 7–60% and that of carcinoma was 11–40%. Of various types of carcinoma, CIS (carcinoma *in situ*) had an incidence of 20%, superficial carcinoma had an incidence of 7–20% and invasive carcinoma had an incidence of 7–20%.

In Group 3 where BBN was administered for 12 weeks, the incidence of dysplasia was 40–100% and that of carcinoma was 20–100% (14–80% for CIS, 20–

60% for superficial carcinoma and 20–57% for invasive carcinoma).

In Group 4 in which BBN was administered for 16 weeks, the incidence of dysplasia was 80–100% and that of carcinoma was 60–100% (38–40% for CIS, 20–63% for superficial carcinoma and 20–88% for invasive carcinoma).

In Group 5 where BBN was administered for 20 weeks, both dysplasia and carcinoma had an incidence of 100%. Of various types of carcinoma, CIS had an incidence of 23–60%, superficial carcinoma had an incidence of 54–90% and invasive carcinoma had an incidence of 40–100%. All mice from Group 6 (untreated controls) had normal bladders.

Experiment 2 (CAP experiment)

Histopathological examination of the bladders yielded the following results (Table 5). In the BBN alone group, the incidence of carcinoma was 18/18 (100%), 2/18 (11%) developed CIS, 10/18 (56%) developed superficial carcinoma and 14/18 (78%) developed invasive carcinoma. In the BBN+CAP treatment group, the incidence of carcinoma in general being 20/27 (74%), CIS, superficial carcinoma, and invasive carcinoma developed in 2/27 (7%), 13/27 (48%), and 7/27 (26%), respectively. The incidence of invasive carcinoma differed significantly between these two groups ($p < 0.025$). In the control groups (Groups 3, 4, and 5), no animal developed tumors.

Experiment 3 (M-VAC, MEC experiment)

Histopathological examination of the bladders yielded the following results (Table 6). Of the 27 mice treated with BBN alone, the incidence of carcinoma was 21/27 (78%), 4/27 (15%) developed CIS, 9/27 (33%) developed superficial carcinoma, and 18/27 (67%) developed invasive carcinoma. In the BBN+M-VAC treatment group, the incidence of carcinoma in general being 19/27 (70%), CIS, superficial carcinoma, and invasive carcinoma was 1/27 (4%), 3/27 (11%), and 14/27 (52%), respectively. In the BBN+low dose MEC treatment group, the incidence of carcinoma in general, CIS, superficial carcinoma, and invasive carcinoma were 11/29 (38%), 6/29 (21%), 7/29 (24%), and 4/29 (14%), respectively. In the BBN+high dose MEC treatment group, the respective incidences were 9/28 (32%), 2/28 (7%), 4/28 (14%), and 4/28 (14%). The incidence of carcinoma

Table 4. Histopathological Findings of the Urinary Bladder in Mice

Group	Subgroup	Period (wks)		Effective No. of mice	Dysplasia	No. of mice with carcinoma	Carcinoma		
		BBN → control					CIS	Super.	Inv.
1	1	4	0	5	0	0	0	0	0
	2	4	4	5	1 (20)	0	0	0	0
	3	4	8	5	1 (20)	0	0	0	0
	4	4	12	5	0	0	0	0	0
	5	4	16	5	0	0	0	0	0
	6	4	20	10	1 (10)	0	0	0	0
	7	4	24	15	0	0	0	0	0
2	1	8	0	5	2 (20)	0	0	0	0
	2	8	4	5	2 (20)	1 (20)	0	1 (20)	0
	3	8	8	5	2 (20)	2 (40)	1 (20)	1 (20)	1 (20)
	4	8	12	10	6 (60)	3 (30)	2 (20)	1 (10)	1 (10)
	5	8	16	9	3 (33)	1 (11)	0	0	1 (11)
	6	8	20	14	1 (7)	2 (14)	0	1 (7)	1 (7)
3	1	12	0	5	2 (40)	1 (20)	0	1 (20)	0
	2	12	4	5	4 (80)	5 (100)	4 (80)	3 (60)	1 (20)
	3	12	8	5	4 (80)	4 (80)	1 (20)	2 (40)	1 (20)
	4	12	12	7	5 (71)	4 (57)	1 (14)	2 (29)	4 (57)
	5	12	16	10	10 (100)	8 (80)	4 (40)	4 (40)	5 (50)
4	1	16	0	5	4 (80)	3 (60)	2 (40)	1 (20)	1 (20)
	2	16	4	5	5 (100)	5 (100)	2 (40)	3 (60)	3 (60)
	3	16	8	8	8 (100)	8 (100)	3 (38)	4 (50)	7 (88)
	4	16	12	8	7 (88)	8 (100)	3 (38)	5 (63)	7 (88)
5	1	20	0	10	10 (100)	10 (100)	5 (50)	9 (90)	4 (40)
	2	20	4	10	10 (100)	10 (100)	6 (60)	7 (70)	7 (70)
	3	20	8	13	13 (100)	13 (100)	3 (23)	7 (54)	13 (100)
6	1	0	4	5	0	0	0	0	0
	2	0	8	5	0	0	0	0	0
	3	0	12	5	0	0	0	0	0
	4	0	16	5	0	0	0	0	0
	5	0	20	5	0	0	0	0	0
	6	0	24	5	0	0	0	0	0
	7	0	28	5	0	0	0	0	0

BBN : N-butyl-N-(4-hydroxybutyl) nitrosamine, CIS : Carcinoma *in situ*,

Super. : Superficial transitional cell carcinoma, Inv. : Invasive transitional cell carcinoma, () : %

Table 5. Histopathological Changes of the Urinary Bladder in Mice (CAP exp.)

Group	Treatment	No. of mice	Effective No. of mice	No. of mice with carcinoma (%)	Carcinoma (%)		
					CIS	Superficial	Invasive
1	BBN alone	22	18	18 (100)	2 (11)	10 (56)	14 (78)
2	BBN → CAP	30	27	20 (74)	2 (7)	13 (48)	7*(26)
3	CAP alone	30	30	0 (0)	0 (0)	0 (0)	0 (0)
4	Saline	13	13	0 (0)	0 (0)	0 (0)	0 (0)
5	Control	10	10	0 (0)	0 (0)	0 (0)	0 (0)

* $p < 0.025$ (Group 1 versus group 2, χ^2 test)

Table 6. Histopathological Changes of the Urinary Bladder in Mice (M-VAC, MEC exp.)

Group	Treatment	No. of mice	Effective No. of mice	No. of mice with carcinoma (%)	Carcinoma (%)		
					CIS	Superficial	Invasive
1	BBN alone	30	27	21 (78)	4 (15)	9 (33)	18 (67)
2	BBN → M-VAC	30	27	19 (70)	1 (4)	3 (11) ^a	14 (52)
3	BBN → MEC (Low)	30	29	11 (38) ^{b,c}	6 (21)	7 (24)	4 (14) ^{d,e}
4	BBN → MEC (High)	30	28	9 (32) ^{f,g}	2 (7)	4 (14) ^h	4 (14) ^{d,e}
5	M-VAC alone	17	17	0 (0)	0 (0)	0 (0)	0 (0)
6	MEC (Low) alone	17	17	0 (0)	0 (0)	0 (0)	0 (0)
7	MEC (High) alone	17	17	0 (0)	0 (0)	0 (0)	0 (0)
8	Saline	10	10	0 (0)	0 (0)	0 (0)	0 (0)
9	Control	10	10	0 (0)	0 (0)	0 (0)	0 (0)

^a: $p < 0.05$ (Group 1 versus Group 2, χ^2 test), ^b: $p < 0.005$ (Group 1 versus Group 3), ^c: $p < 0.025$ (Group 2 versus Group 3), ^d: $p < 0.001$ (Group 1 versus Group 4, 5), ^e: $p < 0.005$ (Group 2 versus Group 4, 5), ^f: $p < 0.005$ (Group 1 versus Group 4), ^g: $p < 0.005$ (Group 2 versus Group 4), ^h: $p < 0.1$ (Group 1 versus Group 4)

differed significantly between the BBN group and the BBN+low dose MEC, high dose MEC treatment groups ($p < 0.005$) and between the BBN+M-VAC group and the BBN+low dose MEC, high dose MEC treatment groups ($p < 0.025$, $p < 0.005$). The incidence of superficial carcinoma differed significantly between the BBN group and the BBN+M-VAC, BBN+high dose MEC treatment groups ($p < 0.05$, $p < 0.1$). The incidence of invasive carcinoma differed significantly between the BBN group and BBN+low dose MEC, high dose MEC treatment groups ($p < 0.001$) and between the BBN+M-VAC group and BBN+low dose MEC, high dose MEC treatment groups ($p < 0.005$). In the control groups (Groups 6 through 9), no mouse developed tumor.

Discussion

To create a valid experimental model of human bladder carcinoma, it is necessary that: (1) the carcinogen has its target only in the urinary bladder; (2) the carcinogen is administered via a natural route; (3) carcinogenesis can be induced at a high incidence in short periods of time; (4) the carcinoma induced is histopathologically similar to human bladder carcinoma; and (5) the carcinoma induced can be diagnosed and treated using techniques employed for clinical cases. Induction of bladder carcinoma by treating dogs, medium-sized experimental animals, and smaller rats or mice with BBN is often used to create an experimental model of human bladder carcinoma which satisfies these requirements.

The experimental model, which involves treatment of dogs with BBN, was developed at our laboratory^{6,7}. By adjusting the dose level and dosing period of BBN, it is possible to induce papillary non-invasive carcinoma and non-papillary invasive carcinoma, resembling those of humans, in the urinary bladders of dogs.

However, when mice, which are small experimental animals like rats, are orally treated with BBN, non-papillary bladder carcinoma develops, which differs from the type of carcinoma seen in rats and is similar to non-papillary invasive bladder carcinoma of humans. Differences in BBN-induced bladder carcinoma in mice depending on BBN dose levels and mouse species have also been studied^{8,9}. Our present preliminary study was undertaken on the basis of such background to examine whether or not an experimental model for studying promoters and inhibitors of bladder carcinogenesis could be created by changing the duration of BBN treatment.

When female C3H/He mice were treated with 0.05% BBN for 8 weeks and subsequently observed without any treatment for 8–12 weeks, only about 10–20% of them developed invasive carcinoma or CIS. However, when the mice were treated with BBN for a longer period (12 weeks) and subsequently observed without any treatment until the 28th week, the incidence of CIS and invasive carcinoma rose to 20–80% and 20–60%, respectively. These results suggest that an experiment involving 8-week BBN treatment is useful in exploring promoters for bladder carcinogenesis in mice, and that an experiment involving

12-week BBN treatment is useful in exploring inhibitors.

Our detailed histopathological examination of the manner of tumor development and progression in mouse bladders revealed that treatment with BBN first causes thickness of the bladder mucosa (i.e., development of simple hyperplasia), and that this is followed by the development of dysplasia. Bladder dysplasia has been thought to develop into CIS and then into non-papillary invasive carcinoma, although it is also possible that dysplasia sometimes shows papillary growth and advances into papillary invasive carcinoma.

Total cystectomy is often used for surgical treatment of human invasive bladder carcinoma. However, treatment using surgery alone does not yield satisfactory results. For this reason, multi-disciplinary therapy, using a combination of radical surgery and adjuvant therapy, including chemotherapy, immunotherapy, and radiotherapy, has been used. Preoperative adjuvant therapy is primarily intended to: (1) reduce the size or stage of tumors as far as possible so that subsequent surgery can be performed more easily, (2) reduce the viability of tumor cells so that local or systemic spread of tumors can be prevented, and (3) treat micrometastasis which may be present¹⁰. Postoperative adjuvant therapy should be provided as far as possible, if we consider that surgical manipulations can induce the spread of tumor cells.

In the present study, a mouse model of BBN-induced bladder carcinoma was created in Experiment 1. Using this model, the effects of three regimens of combination chemotherapy (CAP, M-VAC and MEC regimens) in suppressing the development of bladder carcinoma were examined (Experiments 2 and 3).

As compared to the animals in BBN alone groups, the animals treated with BBN and anti-cancer agents had significantly lower incidences of invasive carcinoma in Experiment 2 and significantly lower incidences of invasive carcinoma or superficial carcinoma in Experiment 3, suggesting that these chemotherapeutic regimens are effective in suppressing the development of invasive bladder carcinoma.

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References

1. Babaya, K, Takahashi, S, Momose, H, Matsuki, H, Sasaki, K, Samma, S, Ozono, S, Hirao, Y, Okajima, E: Effects of single chemotherapeutic agents on development of urinary bladder tumor induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in rats. *Urol Res* **15**: 329-334, 1987.
2. Ozono, S, Iwata, H, Tsunemi, K, Takahashi, S, Matsuki, H, Sasaki, K, Hirao, Y, Okajima, E: Effect of 1-hexyl-carbamoyl-5-fluorouracil on development of rat urinary bladder tumor induced by N-butyl-N-(4-hydroxybutyl) nitrosamine. *Jap J Exp Med* **58**(3): 123-127, 1988.
3. Ozono, S, Babaya, K, Sasaki, K, Okamoto, S, Momose, H, Fujimoto, K, Tsumatani, K, Yamaguchi, H, Hirao, Y, Okajima, E: Rat urinary bladder carcinomas induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine and N-methyl-N-nitrosourea. *Urol Res* **18**: 323-326, 1990.
4. Okajima, E, Ozono, S: Experimental bladder tumor. *Jpn J Urol* **82**: 705-715, 1991.
5. Hayashi, Y, Ozono, S, Yamaguchi, H, Kitagawa, H, Tsunemi, K, Tabata, S, Matsuki, H, Samma, S, Hirao, Y, Okajima, E: Effects of combination chemotherapy including cyclophosphamide, THP-adriamycin, and cisplatin on the development of urinary bladder carcinoma induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in rats. *J Toxicol Pathol* **5**: 61-66, 1992.
6. Okajima, E, Hiramatsu, T, Hirao, K, Ijuin, M, Hirao, Y, Babaya, K, Ikuma, S, Ohara, S, Shiomi, T, Hijioka, T, Ohishi, H: Urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine in dogs. *Cancer Res* **41**: 1958-1966, 1981.
7. Samma, S, Uemura, H, Tabata, S, Iwai, A, Nakatsuji, F, Matsuki, H, Babaya, K, Hirao, Y, Okajima, E: Rapid induction of carcinoma *in situ* in dog urinary bladder by sequential treatment with N-methyl-N'-nitrosourea and N-butyl-N-(4-hydroxybutyl)nitrosamine. *Gann* **75**: 385-387, 1984.
8. Ohtani, M, Kakizoe, T, Nishio, Y, Sato, S, Sugimura, T, Fukushima, S, Nijima, T: Sequential changes of mouse bladder epithelium during induction of invasive carcinomas by N-butyl-N-(4-hydroxybutyl)-nitrosamine. *Cancer Res* **46**: 2001-2004, 1986.
9. Ohtani, M, Kakizoe, T, Sato, S, Sugimura, T, Fukushima, S: Strain differences in mice with invasive bladder carcinomas induced by N-butyl-N-(4-hydroxybutyl)nitrosamine. *J Cancer Res Clin Oncol* **112**: 107-110, 1986.
10. Okajima, E, Ozono, S, Hirao, Y, Babaya, K, Aoyama, H, Maruyama, Y, Samma, S, Iwai, A, Momose, H: Neoadjuvant therapy for locally invasive bladder cancer. *Urol Int* **44**: 332-337, 1989.