patients undergoing radical cystectomy. However, while about half of patients are cured, the remainder still suffer from local recurrence and distant metastasis within 2–3 years. Thus, in an attempt to improve treatment outcome, many investigators have tried combinations of neoadjuvant or adjuvant chemotherapy with surgery (3–5). Unfortunately, the impact of neoadjuvant or adjuvant chemotherapy on survival remains controversial. Recently, the South Western Oncology Group (SWOG) showed an improvement in overall survival with three cycles of neoadjuvant chemotherapy consisting of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) (6). Furthermore, more recent meta-analysis demonstrated that neoadjuvant chemotherapy provided a significant survival advantage in patients with invasive bladder cancer (7).

In this study, we evaluate outcomes of patients with invasive bladder cancer who underwent radical cystectomy with/without pelvic lymph node dissection in 21 hospitals.

PATIENTS AND METHODS

This study included 518 patients with clinically invasive bladder cancer without regional lymph node or distant metastases (T2–4N0M0). All were treated with radical cystectomy with/without pelvic lymph node dissection at 21 hospitals between 1991 and 1995. Using these data, non-randomized, multi-institutional pooled data were analyzed to evaluate the treatment results of radical cystectomy. Tumors were staged according to the criteria of the 3rd edition of General Rules for Clinical and Pathological Studies on Bladder Cancer of the Japanese Urological Association and Japanese Society of Pathology (8). Urothelial carcinoma was the predominant histological type in all patients. Patients with pure squamous cell carcinoma and adenocarcinoma were excluded from this study. Because the pathology of surgical specimens was not reviewed by central pathologist(s), tumor grade was not included in this analysis.

Almost half of the patients received some type of neoadjuvant and/or adjuvant therapy. The type and dose of the additional therapy depended on each institution's preference.

The overall survival was calculated from the date of operation to death from any cause. The overall survival rate was calculated by the Kaplan–Meier method. The statistical significance of differences was determined by the log-rank test. Spearman's rank correlation test was used to analyze correlations between two factors. A P-value of <0.05 was considered statistically significant. All analyses were performed using StatView 5.0 for Macintosh (SAS Institute, NC, USA).

RESULTS

Patient Characteristics

Patient characteristics are shown in Table 1. More than two-thirds of the patients were male. The mean age at operation was 65.4 years (range, 33–87 years). Half of the patients had a clinical stage of T2N0M0. Pathological examination revealed that patients with pT2 and pT3 accounted for almost 60% of the total, followed by those with pT1 and lower stages and those with pT4. Nearly 90% of patients received lymph node dissection. Lymph node metastasis was histopathologically proven in 86 patients (16.6%), who accounted for 18.4% of those who received node dissection (Table 2). Its incidence was significantly linked with clinical stage ($P < 0.01$ by Spearman's rank correlation test). The incidence clearly increased with progression of the pathological stage from 5.9% in patients with superficial cancer to 32.5% of those with pT4 ($P < 0.01$ by Spearman's rank correlation test).

Table 1. Patient characteristics

Characteristics		No. of patients (%)
Gender	Male	400 (77.2)
	Female	118 (22.8)
Age (years)	33–87 (mean: 65.4)	
Clinical T classification	T2	271 (52.3)
	T3	178 (34.4)
	T4	69 (13.3)
Pathological T classification	≤pT1	119 (23.0)
	pT2	156 (30.2)
	pT3	152 (29.4)
	pT4	90 (17.4)
Lymph node metastasis	pNx	53 (10.2)
	pN0	379 (73.2)
	≥pN1	86 (16.6)
Additional therapy	No	268 (51.7)
	Yes	250 (48.3)
Type of additional therapy	Neoadjuvant	118 (47.2)
	Adjuvant	85 (34.0)
	Neoadjuvant and adjuvant	47 (18.8)

Neoadjuvant and/or adjuvant therapies were performed for 48.3% of 518 patients together with radical cystectomy (Table 3). Of these, 118 patients (47.2%) received some type of therapy in the neoadjuvant setting. These included systemic chemotherapy for 80 patients, intraarterial chemotherapy for 32, radiation for one and combined systemic chemotherapy and local radiation for five. Among the systemic chemotherapies, MVAC, the most popular regimen for urothelial cancer (9), was frequently used. In the adjuvant setting, systemic chemotherapy was administered most frequently. More than half of the patients received MVAC chemotherapy.

Outcome

The follow-up period ranged from 0.1 to 11.4 years with a median of 4.4 years. The 5-year overall survival rate was 58% for all 518 patients (Fig. 1), 67% for patients with clinical T2N0M0, 52% for those with T3N0M0 and 38% for those with T4N0M0 (Fig. 2). According to pathological stage, the 5-year

Table 2. Relationships among clinical stage, pathological stage and lymph node metastasis

Clinical stage	Pathological stage	No. of patients with radical cystectomy	No. of pathologically node positive patients/no. of patients with node dissection (%)
T2	pT0	26	1/24 (4.1)
	≤pT1	54	4/48 (8.3)
	pT2	110	8/101 (7.9)
	pT3	57	20/53 (37.7)
	pT4	23	6/19 (31.5)
	All	270	39/245 (15.9)
T3	pT0	7	0/4 (0)
	≤pT1	23	2/18 (11.1)
	pT2	41	2/36 (5.5)
	pT3	78	15/71 (21.1)
	pT4	29	9/28 (32.1)
	All	178	28/157 (17.8)
T4	pT0	5	0/5 (0)
	≤pT1	4	0/3 (0)
	pT2	5	2/5 (40.0)
	pT3	17	5/16 (31.2)
	pT4	38	12/36 (33.3)
	All	69	19/65 (29.2)
T2–4	≤pT1	119	7/119 (5.9)
	pT2	156	12/142 (8.4)
	pT3	152	40/140 (28.5)
	pT4	90	27/83 (32.5)

$P < 0.01$ (Spearman's rank correlation test).

Table 3. Type of additional therapy

Type		No. of courses (median)	No. of patients
Neoadjuvant			118
Systemic chemotherapy	MVAC*	1–4 (2)	49
	MEC*	1–4 (2)	13
	CDDP-based chemotherapy	1–2 (2)	18
Local therapy	Intraarterial chemotherapy (CDDP-based)	1–2 (1)	32
	Radiation only		1
Systemic and local therapy	Chemotherapy and radiation		5
Adjuvant			85
Systemic chemotherapy	MVAC	1–4 (2)	48
	CISCA*	1–3 (2)	5
	MEC	1–2 (2)	4
	CDDP-based chemotherapy	1–6 (2)	24
	Others		4
Neoadjuvant and adjuvant			47
Intraarterial→systemic			13
Systemic and radiation→systemic			4
Systemic→systemic			30

*MVAC, methotrexate, vincristine, doxorubicin and cisplatin, (21); MEC, methotrexate, epirubicin and cisplatin, (22); CISCA, cisplatin, cyclophosphamide and doxorubicin.

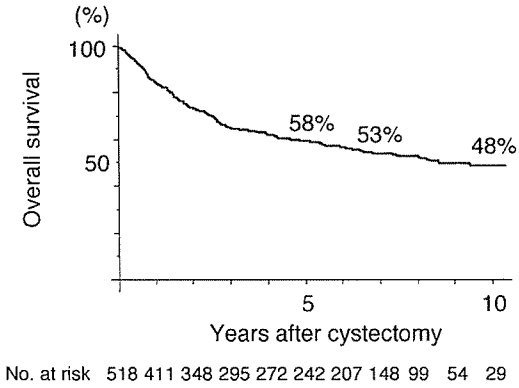

Figure 1. Overall survival rate in all 518 patients.

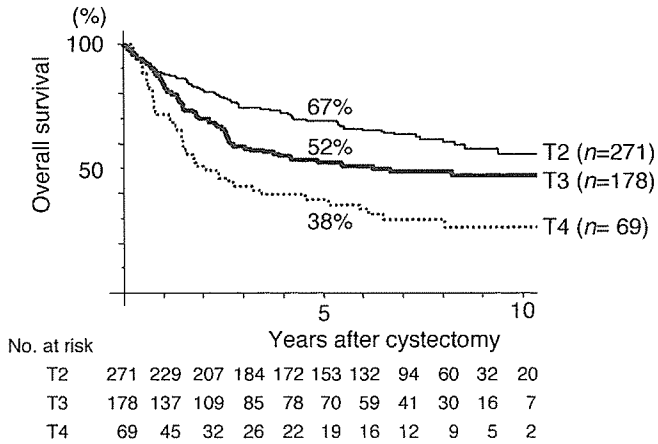

Figure 2. Overall survival rate according to clinical stage. T2 versus T3, $P < 0.01$; T2 versus T4, $P < 0.001$; T3 versus T3, $P < 0.01$ (log-rank test).

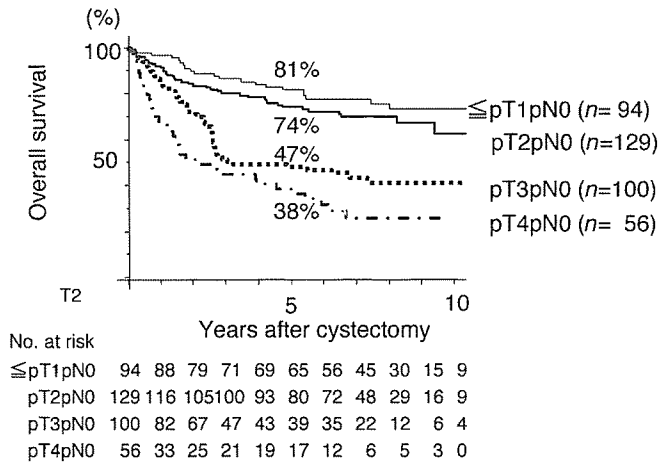

Figure 3. Overall survival rate according to pathological stage. ≤pT1pN0 versus pT3pN0, pT4pN0, $P < 0.001$; pT2pN0 versus pT3pN0, pT4pN0, $P < 0.001$; pT3pN0 versus pT4pN0, $P = 0.02$ (log-rank test).

overall survival rate was significantly higher for patients with pT1 or a lower stage, or pT2 than for those with pT3 or pT4 disease, when those who were pathologically node negative were considered (Fig. 3). Patients who were pathologically proven to be node positive clearly had a lower 5-year overall survival rate (30%) than those who were node negative (Fig. 4, $P < 0.001$ by log-rank test).

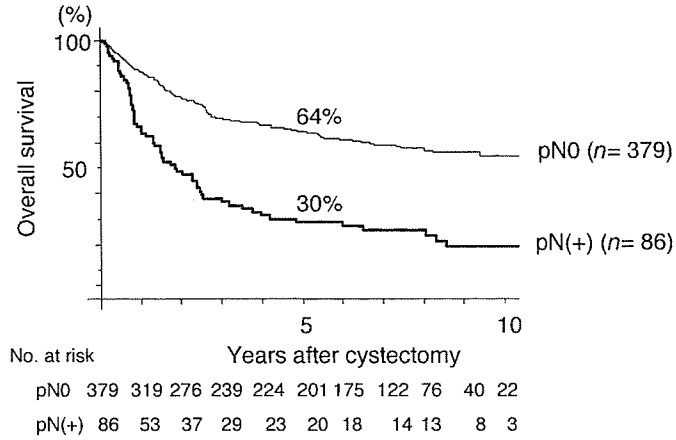

Figure 4. Overall survival rate according to lymph node metastasis. pN0 versus pN(+), $P < 0.001$ (log-rank test).

Impact of Additional Therapy

When we evaluated whether neoadjuvant chemotherapy could improve survival, there was no significant difference with regard to the 5-year overall survival between patients with and without the therapy (65% versus 56%, $P = 0.13$ by log-rank test) (Fig. 5). Furthermore, neoadjuvant chemotherapy did not influence the overall survival among all clinical stages. Similarly, adjuvant chemotherapy did not improve the prognosis because the 5-year overall survival rates for all patients with and without this therapy were 57% and 56%, respectively. When we investigated the influence of adjuvant chemotherapy on the 5-year overall survival in patients with pT2 or a lower stage without lymph node metastasis, there was no significant difference between patients with and without the therapy. No survival benefit was found for the therapy in patients with pT3 or pT4 without pathologically proven lymph node metastasis. However, the therapy improved the 5-year overall survival in patients with lymph node metastasis, with a significant difference between those with and without it ($P < 0.001$, by log-rank test) (Fig. 6).

DISCUSSION

In this study we evaluated the treatment outcomes of patients with invasive bladder cancer who underwent radical cystectomy with/without pelvic lymph node dissection in 21 hospitals from 1991 to 1995. The study enabled us to analyze the 5-year survival rates of a large volume of patients. The analysis showed that the 5-year overall survival rate for patients with T2N0M0, T3N0M0 and T4N0M0 tumors were 67%, 52% and 38%, respectively. These results are similar to/better than a previous report that the 5-year survival rates were 49% (95% CI: 39–59%) for patients with T2, 25% (95% CI: 10–50%) for those with T3 and 17% (95% CI: 5–45%) for those with T4,

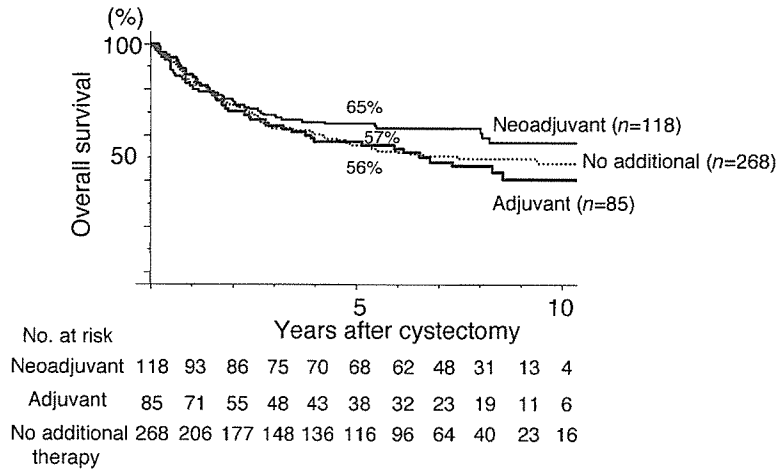

Figure 5. Overall survival rate according to additional therapy. Neoadjuvant versus no additional therapy, $P = 0.13$ (log-rank test); adjuvant versus no additional therapy, $P = 0.72$ (log-rank test).

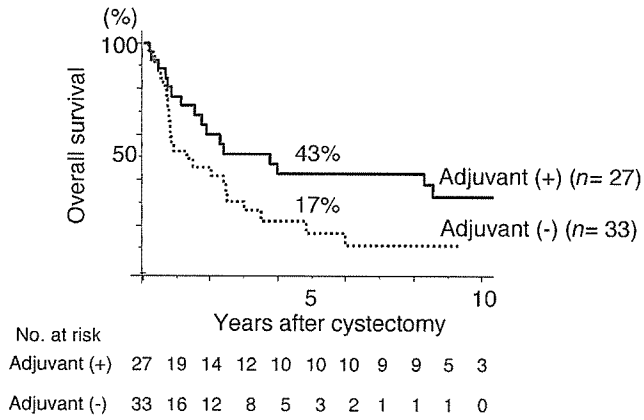

Figure 6. Overall survival rate according to adjuvant therapy in patients with lymph node metastasis. Adjuvant (+) versus adjuvant (–), $P = 0.03$ (log-rank test).

although this report was published 10 years ago (10). Similarly, the analysis according to pathological stage revealed results consistent with those in previous studies showing that the 5-year survival was 76–85% for pT1 or lower stage, 64–84% for pT2pN0, 25–56% for pT3pN0 and 19–44% for pT4pN0 (1,11,12). In Japan, the analysis of 351 patients who underwent radical cystectomy at a single institute showed a similar result (13).

In the present study pathologically proven lymph node metastasis was seen in 18% of patients with lymph node dissection. Some reports indicated that lymph node metastasis was present in 15–34% of patients who underwent radical cystectomy (10,14–16). The variation in the incidence of positive nodes may stem from the heterogeneous profiles of patients, extent of lymph dissection, and the number of lymph nodes removed. Indeed, Leissner et al. (14) reported a correlation between the number of lymph nodes removed (≥16 lymph nodes) and the percentage of patients with positive nodes, especially in locally advanced bladder cancer. Lymph node metastasis is reported to be an independent poor prognostic factor (14–16). Our study supported previous results since the present study also showed that patients with positive nodes had a worse prognosis. Recently, the number of positive lymph nodes, rather than the size, was reported to be associated with death from bladder cancer (15,16). Unfortunately we did not assess the number of lymph nodes in this study. Further study will be necessary to confirm these results. At present it remains controversial whether lymph node dissection has a therapeutic effect or is merely a staging tool. Some investigators advocate extensive bilateral lymphadenectomy as a potentially curative procedure (14,16).

Since the 5-year survival rate with radical cystectomy alone seems to reach a plateau, especially in patients with locally advanced bladder cancer, various trials of additional treatments before and/or after surgery have been carried out (3–5). Unfortunately, it remains undefined whether neoadjuvant or adjuvant chemotherapy with surgery improves the survival (17). However, in the SWOG study, patients with three cycles of neoadjuvant MVAC achieved survival benefit with the median survival of 77 months, as compared with 46 months among patients with surgery alone, although the difference was not significant when it was analyzed by a two-sided stratified log-rank test (6). Furthermore, more recent meta-analysis demonstrated that neoadjuvant cisplatin-based combination chemotherapy provided a survival advantage over a definitive local therapy (7). Our group started a prospective phase III study evaluating the survival benefit of two cycles of MVAC followed by surgery over surgery alone in patients with T2–4N0M0 bladder cancer with the support of the Japanese Clinical Oncology Group.

On the other hand, our retrospective study showed that patients with lymph node metastasis had a survival benefit from adjuvant chemotherapy, although only a small number of patients were included. Some investigators also reported the impact of adjuvant chemotherapy on survival of these patients in retrospective studies (15,16). Furthermore, prospective studies demonstrated a significant survival benefit (18–20). However, these studies were criticized due to their small

numbers of patients, early termination of trials and confusing methodology for analysis. Therefore, the role of adjuvant chemotherapy remains a matter of debate. To evaluate the impact of immediate adjuvant chemotherapy after cystectomy, the European Organization for Research and Treatment of Cancer has launched a large randomized trial that plans to enroll 1344 patients. In the near future its results will tell us whether immediate adjuvant chemotherapy is necessary in high-risk patients.

In summary, our retrospective, multi-institutional analysis showed that radical cystectomy provided an overall survival for patients with clinically invasive bladder cancer similar to that of previous reports. Thus, it is clear that surgery alone will not provide better survival than we have now. Therefore, additional therapy is mandatory to improve the treatment outcome. Further large-scale randomized studies will be needed to clarify the timing and type of additional therapy.

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References

1. Schoenberg M. Management of invasive and metastatic bladder cancer. In Walsh PC, et al., editors. Campbell's Urology, 8th edition. Philadelphia: Saunders, 2002;2803–17.
2. Carrion R, Seigne J. Surgical management of bladder carcinoma. *Cancer Control* 2002;9:284–92.
3. Dreicer R. Neoadjuvant chemotherapy in the management of muscle-invasive bladder cancer. *Semin Urol Oncol* 2001;19:180–85.
4. Lehmann J, Retz M, Stockle M. The role of adjuvant chemotherapy for locally advanced bladder cancer. *World J Urol* 2001;19:133–40.
5. Sternberg CN. Neo-adjuvant and adjuvant chemotherapy of bladder cancer: Is there a role? *Ann Oncol* 2002;13(Suppl 4):273–9.
6. Grossman HB, Natale RB, Tangen CM, Speights VO, Vogelzang NJ, Trump DL, et al. Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med* 2003;349:859–66.
7. Advanced Bladder Cancer Meta-analysis Collaboration. Neoadjuvant chemotherapy in invasive bladder cancer: a systematic review and meta-analysis. *Lancet* 2003;361:1927.
8. Japanese Urological Association, Japanese Society of Pathology. General Rules for Clinical and Pathological Studies on Bladder Cancer, 3rd edition. Tokyo: Kanehara 2001 (in Japanese).
9. Sternberg CN, Yagoda A, Scher HI, Watson RC, Geller N, Herr HW, et al. Methotrexate, vinblastine, doxorubicin and cisplatin for advanced transitional cell carcinoma of the urothelium. Efficacy and patterns of response and relapse. *Cancer* 1989;64:2448–58.
10. Paulson DF. Critical review of radical cystectomy and indicators of prognosis. *Sem Urol* 1993;11:205–13.
11. Dalbagni G, Genega E, Hashibe M, Zhang ZF, Russo P, Herr H, et al. Cystectomy for bladder cancer: a contemporary series. *J Urol* 2001;165: 1111–6.
12. Stein JP, Lieskovsky G, Cote R, Groshen S, Feng AC, Boyd S, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. *J Clin Oncol* 2001;19:666–75.
13. Kuroda M, Meguro N, Maeda O, Saiki S, Kinouchi T, Usami M, Kotake T. Stage specific follow-up strategy after cystectomy for carcinoma of the bladder. *Int J Urol* 2002;9:129–33.
14. Leissner J, Hohenfellner R, Thuroff JW, Wolf HK. Lymphadenectomy in patients with transitional cell carcinoma of the urinary bladder; significance for staging and prognosis. *BJU Int* 2000;85:817–23.
15. Stein JP, Cai J, Groshen S, Skinner DG. Risk factors for patients with pelvic lymph node metastases following radical cystectomy with en bloc pelvic lymphadenectomy: concept of lymph node density. *J Urol* 2003; 170:35–41.
16. Frank I, Cheville JC, Blute ML, Lohse CM, Nehra A, Weaver AL, et al. Transitional cell carcinoma of the urinary bladder with regional lymph node involvement treated by cystectomy: clinicopathologic features associated with outcome. *Cancer* 2003;97:2425–31.
17. Juffs HG, Moore MJ, Tannock IF. The role of systemic chemotherapy in the management of muscle-invasive bladder cancer. *Lancet Oncol* 2002;3: 738–47.
18. Skinner DG, Daniels JR, Russell CA, Lieskovsky G, Boyd SD, Nichols P, et al. The role of adjuvant chemotherapy following cystectomy for invasive bladder cancer: a prospective comparative trial. *J Urol* 1991;145:459–64.
19. Stockle M, Meyenburg W, Wellek S, Voges GE, Rossmann M, Gertenbach U, et al. Adjuvant polychemotherapy of nonorgan-confined bladder cancer after radical cystectomy revisited: long-term results of a controlled prospective study and further clinical experience. *J Urol* 1995;153:47–52.
20. Freiha F, Reese J, Torti FM. A randomized trial of radical cystectomy versus radical cystectomy plus cisplatin, vinblastine and methotrexate chemotherapy for muscle invasive bladder cancer. *J Urol* 1996;155: 495–9.
21. Kuroda M, Kotake T, Akaza H, Hinotsu S, Kakizoe T. Efficacy of dose-intensified MEC (Methotrexate, Epirubicin, Cisplatin) chemotherapy for advanced urothelial carcinoma: a prospective randomized trial comparing MEC and M-VAC (Methotrexate, Vinblastine, Doxorubicin and Cisplatin). *Jpn J Clin Oncol* 1998;28:497–501.
22. Lynch DF Jr. Preoperative and postoperative adjuvant chemotherapy using CISCA (cyclophosphamide, doxorubicin, and cisplatin) in the treatment of invasive transitional-cell carcinoma of the bladder. *Urol Clin North Am* 1991;18:543–6.

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The Same-Pedicle Concept for Continent Urinary Diversion Using a Yang-Monti Reconfigured Tube

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Key Words

Continent valve · Diversion, urinary · Reservoir, urinary · Yang-Monti tube

Abstract

Objective: To facilitate the anastomosis of a continent valve to the umbilicus or a suitable skin area, we used a reconfigured tube made from the same segment of the intestinal reservoir as that used to construct the urinary pouch. ***Materials and Methods:*** Seven patients underwent continent ileal-pouch formation using a reconfigured ileal tube following cystectomy for bladder cancer. Two irradiated patients and 1 patient with neurogenic bladder underwent continent colon-pouch construction with a reconfigured colon tube. ***Results:*** The average length of the reconfigured ileal tube was 5 cm, while the colon tube was maximally 10 cm long. All procedures were technically straightforward. All the continent pouches functioned well, without catheterization difficulties. ***Conclusions:*** Since the Yang-Monti tube and the pouch are easily mobilized, being based on the same vascular pedicle, and can therefore bridge the gap, making the umbilical anastomosis was greatly facilitated. Sufficient support for the tube is provided by the pouch.

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Introduction

An innovative procedure in which a short ileal segment is reconfigured transversally to create a tubular structure for urologic reconstruction was developed by Yang [1] and Monti et al. [2]. Immediately, this was appreciated as a new second-line Mitrofanoff tube for the construction of a continent valve as an alternative to the vermiform appendix. With several advantages (constant availability, minimal loss of bowel, no interference with the mesentery and high mobility of the tube), this technique is actually an excellent alternative to the use of the Mitrofanoff principle [3]. However, it does have one limitation: namely, the tube length is determined by the circumference of the ileal segment. This becomes especially important when the tube is to be used either for a continent ileovesicostomy in obese patients or to bridge the gap between the native bladder and the umbilicus over a long distance. To overcome this problem, a double-length tube can be formed from separate ileal segments [3] or from a continuous ileal segment [4]. Alternatively, a transverse retubularized sigmoid-colon segment is a good choice for the purpose of vesicostomy using an intestinal segment [5].

Another solution to the problem of how to anastomose a continent reconfigured ileal tube to a desirable skin location or to the umbilicus is to implant it into an intestinal segment for augmentation, thus effectively extending it so as to bridge the gap if this is indicated [3]. For this purpose, a reconfigured ileal segment seems to be preferable

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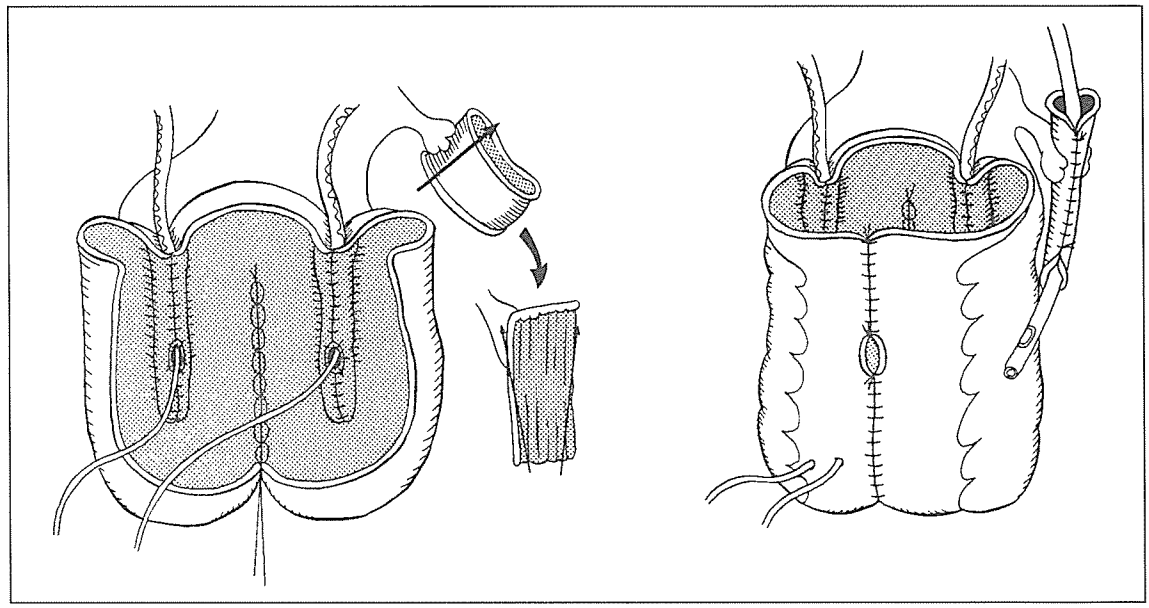

Fig. 1. After ureteral implantation, a short ileal plate is formed by opening a small ileal segment and cutting the edges obliquely. An anterior wall is created with one mucosal hole, and the ileal plate is retubularized to make a cone-shaped tube.

to the appendix because of the proximity of the former to the pouch and the adequate mobility of its vascular pedicle [6]. Having recognized the advantages of the use a reconfigured ileal tube connected to the same mesenteric pedicle as the pouch, we adapted the original idea aggressively to reconstructive surgery of the urinary tract, and we have now evaluated this modified procedure.

Materials and Methods

In 7 patients with an invasive bladder cancer (mean age 52 years, range 38–63), a continent ileal pouch with an umbilical stoma was constructed after radical cystectomy. As shown in figure 1, to construct a continent tube, an additional 3-cm-long segment obtained from the oral end of the pouch was opened longitudinally 1 cm from the mesentery to create an ileal plate, and the side edges of the plate were tailored to form a slim trapezium. The small ileal plate was retubularized over a 14-french catheter in the transverse direction to create a cone-shaped tube, which was divided into a short and a long portion by the mesentery (fig. 1). After ureteric implantation, the end of the long portion was anastomosed to the mucosal hole in the anterior pouch, while the long portion was embedded in a serous-lined tunnel with the transverse suture line of the tube facing the anterior pouch wall (fig.2). Finally, the end of the short portion of the ileal tube was anastomosed to a skin hole made at the bottom of the umbilicus. In 5 of these patients a healthy appendix was present, and we left it in place.

In 2 patients who had undergone pelvic irradiation (57 and 60 years old, respectively), a continent transverse colon pouch was constructed. To make a continent tube, a short colon segment cut from the main colon segment used for pouch construction was reconfigured transversally over a 14-french catheter. The reconfigured tube was implanted submucosally into the anterior pouch, and the distal end was connected to a skin opening at the umbilicus or at a suitable skin location (fig. 3). In 1 patient with a neurogenic bladder due to spina bifida (12 years old), we performed augmentation cystoplasty using a sigmoid colon with construction of a continent umbilical stoma. Since the appendix was used for a Malone antegrade continent enema stoma [7], a short colon segment was taken from the main segment used for the augmentation. This short segment was reconfigured to make a continent tube, which was embedded into a submucosal tunnel created in the augmenting flap (fig. 4). The distal end was connected to a skin opening at the umbilicus.

In the 3 patients in whom a colon segment was used to create a continent tube, the segment was opened at its antimesenteric border, and was thus divided into two portions of equal length by the mesenteric pedicle. For anastomosis to the umbilicus or a suitable skin site, the free end of the reconfigured tube was trimmed to the required length, since we always fixed the pouch wall to the back of the abdominal muscle in order to make the distance short and to avoid angulation and possible kinking of the tube.

Results

The length of the reconfigured ileal tube averaged about 5 cm (4–6 cm) and that of the colon tube about 9 cm (8–10 cm). All tubes provided a sufficient length for implantation either into a serous-lined tunnel in an ileal

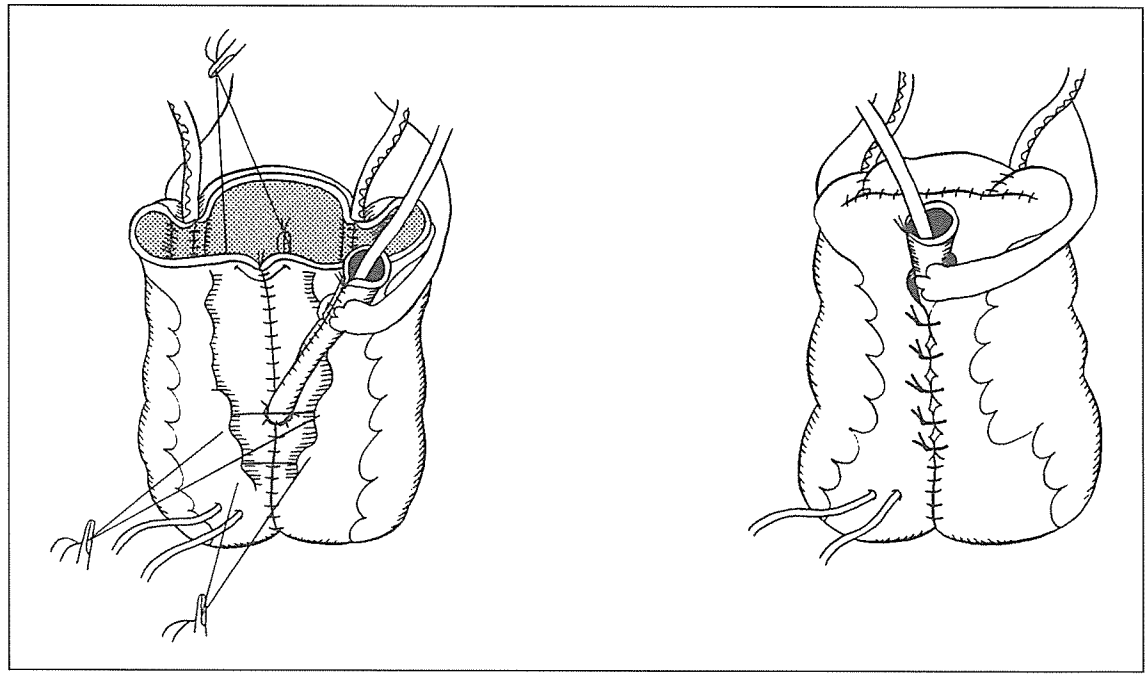

Fig. 2. The tube is anastomosed to the mucosal hole and then embedded into a serous-lined tunnel in the anterior pouch wall. The transverse suture line of the tube faces the pouch wall.

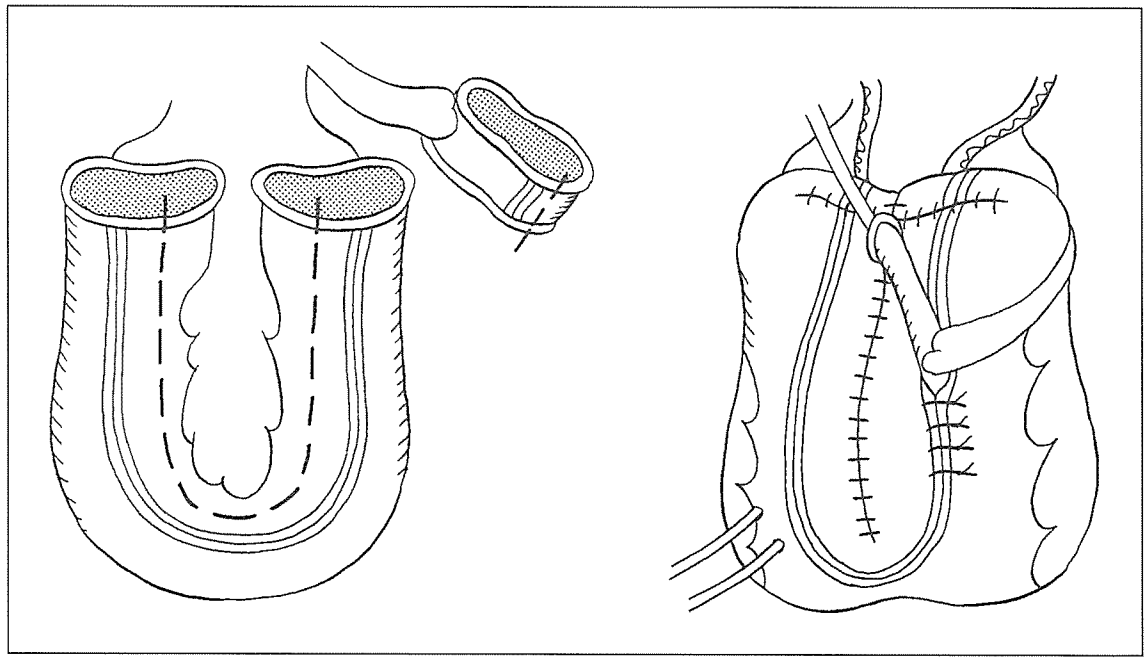

Fig. 3. The transverse colon segment for the pouch and an additional short segment are taken from one and the same pedicle. The retubularized tube made from the short segment is implanted into a submucosal tunnel in the pouch wall.

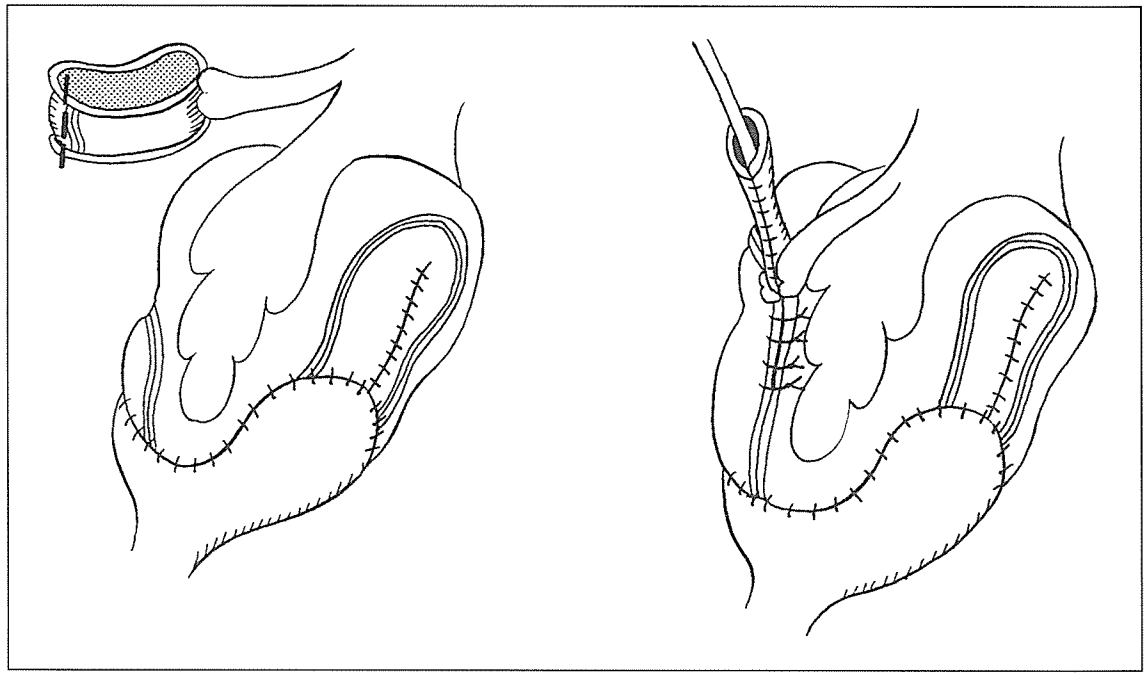

Fig. 4. The native contracted bladder is augmented by a segment of sigmoid colon. An additional short segment isolated from the same pedicle is retubularized and implanted submucosally into the flap augmenting the native bladder.

pouch or into a submucosal tunnel in a colon pouch. The distal end of the tube easily reached far enough to be anastomosed to a hole at the bottom of the umbilicus or in a suitable area of the skin.

With a mean follow-up of 35 (8–56) months, all patients were evaluated by pouchography and intravenous urography 3 and 6 months after surgery. Two patients with a continent ileal pouch died due to cancer progression during the follow-up (8 and 12 months after surgery, respectively). All upper urinary tracts were stable on intravenous urography. All patients could catheterize the pouch easily using a 14-french catheter, and they all had a functional pouch capacity of more than 400 ml at 6 months (range, 400–700 ml). No complications were experienced after construction of the pouch (e.g. stoma-related problems) in any of the patients.

Discussion

Refashioning a small ileal segment transversally to form a Mitrofanoff continent tube was originally described by Yang [1] and Monti et al. [2]. We personally recognized this Yang-Monti method to be geometrically well designed and practically useful, since it could avoid problems inherent in the use of either the appendix or a tapered ileum [6].

However, in general, this technique has been thought of as a second-line Mitrofanoff tube alternative to the vermiform appendix. Only when the appendix was absent or unsuitable for use as a continent tube [3, 8, 9], or when it was planned to use the appendix for a Malone antegrade colonic enema [9], was the Yang-Monti technique applied for the creation of a continent tube.

Although we believe that the mobility of the reconfigured ileal tube on the vascular pedicle is superior to that of the appendix, one limitation of the Yang-Monti technique is the shortness of the tube, which is determined by the circumference of the ileal segment. The values given in the literature for the length of the reconfigured ileal tube are 6–7 cm [3, 9], or 8–13 cm if a colon segment is included [8]. Unfortunately, in our Japanese patients the value is only about 5 cm, not 6–7 cm. Consequently, it could prove difficult for us to bridge the gap between the reservoir and the skin stoma. To overcome this type of problem, other authors have formed a double tube from two retubularized ileal segments joined together (12 cm long) [3] or from a single piece of ileum (10–14 cm long) [4] for ileovesicostomy. A retubularized sigmoid-colon segment (13 cm long) has also been reported to be effec-

tive at bridging the gap between the bladder and the umbilicus [5].

To minimize complications related to the use of these reconfigured bowel tubes for a continent vesicostomy, the tube should be straight, with no helical rotation and without tension, after the construction. Furthermore, we believe that leaving part of the tube free should be avoided; it should be supported by the bladder or by the reservoir to prevent catheterization problems. Following the construction of Yang-Monti ileovesicostomies, the conduits have been found to have a considerably higher incidence of catheterization problems than the appendix [10]. Narayanaswamy et al. [10] observed that the problems unique to the Yang-Monti channel were a pouch-like dilatation and angulation of the channel in its middle or at its entry into the bladder. The angulation resulted from it being free lying and poorly supported, which could finally cause pouch formation. For these reasons, some investigators have adhered to the appendix as their first choice [3, 8, 9], or else used a reconfigured sigmoid-colon segment instead of an ileal segment [5] for a continent vesicostomy. Thus, an ideal Yang-Monti channel should be straight and supported extramurally with no free portion by the bladder or reservoir to prevent both catheterization problems and dilatation of the tube.

With regard to the indication for construction of a continent vesicostomy using a bowel tube, simultaneous augmentation cystoplasty is commonly required for a small, poorly compliant bladder. Gerharz et al. [3] reported that among their 16 patients, a continent reconfigured ileal valve was created and reimplanted mostly into the submucosal tunnel in an augmented bladder in 12, and into an intestinal reservoir in 1 patient. Gosalbez et al. [8], who also performed concomitant cystoplasty associated with a reconfigured bowel tube for a continent valve, used the ileum in 3 cases and the sigmoid colon in 1. Although they implanted the reconfigured ileal tube into the native bladder, they recognized the advantage of the same-pedicle concept, which shortens the distance to the abdominal wall or umbilicus. They also mentioned that this aspect of the procedure became much less of a problem when a colon segment was used, since the tube was longer and could be implanted submucosally anywhere along the colon patch as well as in the native bladder.

However, we favor the same-pedicle concept, entailing a tubular structure for the continent valve being prepared from the same segment of the bowel as that used for the pouch or for augmentation, so that the tube is implanted into the segment of the bowel from which it originates. In addition to the augmentation effect itself, the pouch or flap used for augmentation greatly narrows the gap to the umbilicus or suitable skin site, and provides sufficient support even if the tube is short, since the mesenteric pedicle to the tube and the tissue used for augmentation may be mobilized together. Furthermore, these effects are greatly enhanced when the Yang-Monti tube is applied using the same-pedicle concept, since the tube can be easily embedded in the pouch wall with no interference with the mesenteric pedicle [6]. To prevent dilatation of the Yang-Monti tube, our practice is to ensure that the transverse suture line of the tube is facing the anterior pouch wall (fig. 2).

Although the Yang-Monti colon tube can be easily embedded into a submucosal tunnel in a colon segment, burying an ileal reconfigured tube into the ileal wall is more difficult, since the submucosal-tunnel technique is not suitable for ileal segments. This might be a reason for implanting the reconfigured ileal tube into the colon segment used for augmentation [3], or into the native bladder, despite the use of an ileal flap [8, 9]. However, recent advances in the serous-lined tunnel principle made by Abol-Enein and Ghoneim [11] enabled us to create an effective continent catheterizable tube in an ileal pouch. Although they used a tapered ileum or the appendix for a continent valve, they successfully established a continent ileal pouch by creating the serous-lined tunnel. Thus, we could overcome the problems and make full use of the benefit of the same-pedicle concept. In addition, with the present method, angulation of the continent tube during catheterization ought to be prevented, since the entrance of the serous-lined tunnel is so natural. Böhme and Tauber [12] reported a similar continent ileal pouch using a retubularized ileal segment embedded in the serous-lined tunnel, and we found the same-pedicle concept in the report by Castellan et al. [13].

As there were no complications related to the present continent tube, despite a relatively long-term follow-up in our patients, we believe that the Yang-Monti bowel tube can be considered a first-line Mitrofanoff tube when the tube is created from the same bowel segment as that used for pouch formation or augmentation.

Conclusion

The Yang-Monti reconfigured tube should be considered instead of the appendix for use as a continent urinary diversion when the tube is obtained from the same segment of the intestine as that used for the pouch or augmentation. This concept provides considerable benefits to urinary reconstruction.

References

1 Yang WH: Yang needle tunneling technique in creating antireflux and continent mechanisms. J Urol 1993;150:830–834.
2 Monti PR, Lala RC, Durta MR, Rezende J, DeCarvalho JR: New technique for construction of efferent conduits based on the Mitrofanoff principle. Urology 1997;49:112–115.
3 Gerharz EW, Tassadaq T, Pickard RS, Shah JR, Woodhouse CRJ, Ransley PG: Transverse retubularized ileum: Early clinical experience with a new second line Mitrofanoff tube. J Urol 1998;159:525–528.
4 Casale AJ: A long continent ileovesicostomy using a single piece of bowel. J Urol 1999;162:1743–1745.
5 Van Savage JG, Yepuri JN: Transverse retubularized sigmoidovesicostomy continent urinary diversion to the umbilicus. J Urol 2001;166:644–647.
6 Kato H, Igawa Y, Kiyokawa H, Iwata K, Nishizawa O: Continent ileal pouch using the serous-lined principle. Eur Urol 2000;37:100–103.
7 Malone PS, Ransley PG, Kiely EM: Preliminary report: The antegrade continent enema. Lancet 1990;336:1217–1218.
8 Gosalbez R, Wei D, Gousse A: Refashioned short bowel segments for the construction of catheterizable channels (the Monti procedure): Eary clinical experience. J Urol 1998;160:1099–1102.
9 Cain MP, Casale AJ, King SJ, Rink RC: Appendicovesicostomy and new alternative for the Mitrofanoff procedure: Results in the last 100 patients at Riley Children's Hospital. J Urol 1999;162:1749–1752.
10 Narayanaswamy B, Wilcox DT, Cuckow PM, Duffy PG, Ransley PG: The Yang-Monti ileovesicostomy: A problematic channel? Br J Urol 2001;87:861–865.
11 Abol-Enein H, Ghoneim MA: Serous-lined extramural ileal valve: A new continent urinary outlet. J Urol 1999;161:786–791.
12 Böhme H, Tauber R: Transverse tubularized segments of ileum as continent efferent conduits to the navel in Hautomann's ileum neobladder. Urologe [A] 2000;39:436–439.
13 Castellan MA, Gosalbez R, Labbie A, Monti PR: Clinical application of the Monti procedure as a continent catheterizable stoma. Urology 1999;54:152–156.

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Nrf2 Is Essential for the Chemopreventive Efficacy of Oltipraz against Urinary Bladder Carcinogenesis

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ABSTRACT

The induction of phase 2 detoxifying enzymes, such as UDP-glucuronosyltransferases (UGTs), in response to an array of naturally occurring and synthetic agents, such as oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione), provides an effective means of protection against a variety of carcinogens. Transcription factor Nrf2 is an essential regulator of the inducible expression of detoxifying enzyme genes by chemopreventive agents. In this study, we investigated in Nrf2-deficient mice the susceptibility to the urinary bladder-specific carcinogen *N*-nitrosobutyl(4-hydroxybutyl)amine (BBN) and the chemopreventive efficacy of oltipraz. The incidence of urinary bladder carcinoma by BBN was significantly higher in *Nrf2*$^{-/-}$ mice than in wild-type mice; invasive carcinoma was found in 24.0 and 38.5% of wild-type and *Nrf2*$^{-/-}$ mice, respectively. Oltipraz induced the phase 2 enzymes responsible for BBN detoxification in the liver and urinary bladder in an Nrf2-dependent manner. As expected, therefore, oltipraz decreased the incidence of urinary bladder carcinoma by BBN in wild-type mice but had little effect in *Nrf2*$^{-/-}$ mice. In wild-type mouse liver, oltipraz significantly induced BBN glucuronidation and decreased the urinary concentration of *N*-nitrosobutyl(3-carboxypropyl)amine, a proximate carcinogen of BBN. Importantly, BBN was found to suppress the expression of *UGT1A* specifically in the urinary bladder. This suppression was counteracted by oltipraz in wild-type mice but not in *Nrf2*$^{-/-}$ mice. These results show that Nrf2 and its downstream target genes are responsible for BBN detoxification. Furthermore, oltipraz prevents carcinogenesis by BBN by enhancing detoxification of this carcinogen in the liver and urinary bladder.

INTRODUCTION

The relationship between chemical exposure and urothelial cancer has been well established since 1895, when it was suggested that men working in the dye industry were at increased risk of bladder cancer (1). 2-Naphthylamine subsequently was determined to be one of the causative agents (2). Currently, cigarette smoking is considered a major risk factor for the development of bladder cancer in industrialized countries (1). Similarly, *N*-nitroso compounds (NOCs) have been proposed as etiologic agents of bladder cancer associated with schistosomiasis (3). NOCs also are thought to play roles in the carcinogenesis of the stomach, esophagus, and pharynx in humans (4). *N*-nitrosodibutylamine, which was first identified as a rat bladder carcinogen, has been detected as a pollutant in tobacco smoke, corrosion inhibitor, food, and rubber products (5). Although it exists in a low concentration, it is considered practically as carcinogenic to humans (6). *N*-nitrosodibutylamine is metabolized mainly in the liver, and tumor induction in the rat bladder seems to depend on the formation of two ω-oxidized metabolites, *N*-nitrosobutyl(4-hydroxybutyl)amine (BBN) and its proximate carcinogen *N*-nitrosobutyl(3-carboxypropyl)amine (BCPN; ref. 7). Oral administration of BBN to rats and mice induces cancer specifically in the urinary bladder (8). BBN-induced urinary bladder carcinogenesis in rodents is an excellent model system to understand the carcinogenic mechanisms by NOC.

Several lines of epidemiologic and experimental evidence suggest that a decreased expression in carcinogen-detoxifying enzymes, such as *N*-acetyl transferase 2 (9, 10), glutathione *S*-transferase (GST) M1 (9, 11), NAD(P)H quinone oxidoreductase (NQO1; ref. 12), and UDP-glucuronosyltransferase (UGT) 1A (13), is associated with urinary bladder cancer. The urinary bladder-specific carcinogenic effect of BBN may result, at least in part, from the metabolic fate of the compound because BCPN, the major urinary metabolite of BBN, has been shown to have carcinogenic effects on urothelial cells (14, 15). Following α-hydroxylation, BCPN and BBN are chemically cleaved to their corresponding alkylcarbonium ion that binds covalently to DNA and enhances carcinogenesis (16).

Carcinogens are normally detoxified by conjugation with water-soluble cofactors. Typical examples of such cofactors are glutathione and glucuronic acid, which are conjugated to carcinogens through the actions of GSTs and UGTs, respectively. These conjugating enzymes have been categorized as phase 2 detoxifying enzymes (17). It has been proposed that induction of phase 2 detoxifying enzyme genes plays a major role in protection against carcinogens (18). A recognized characteristic action of chemopreventive agents, including the phenolic antioxidants 2,3-butyl-4-hydroxyanisole (19) and 1,2-dithiole-3-thione (20) and the isothiocyanates (21), is their potential to induce phase 2 enzymes. Oltipraz (4-methyl-5-[2-pyrazinyl]-1,2-dithiole-3-thione) represents one of the most potent inducers of phase 2 enzymes (22, 23).

The induction of phase 2 enzyme genes is regulated by their *cis*-acting antioxidant response element (ARE) or electrophile responsive element (EpRE; refs. 24–26). Transcriptional factor Nrf2 binds to and regulates transcription through the ARE/EpRE after heterodimerizing with one of the small Maf proteins (27–29). Germline mutant mice specifically lacking the *Nrf2* gene have been established (30, 31). When we treated these mice with 2,3-butyl-4-hydroxyanisole, we found that *Nrf2*$^{-/-}$ mice lack the inducible expression of phase 2 and antioxidant enzymes, providing conclusive evidence for the notion that Nrf2 regulates their transcription (30).

An obvious hypothesis then is that *Nrf2*$^{-/-}$ mice are more susceptible to oxidative and electrophilic stresses, and this hypothesis has been tested in various contexts (32–40). For example, the forestomach tumor formation caused by benzo(*a*)pyrene is markedly increased in *Nrf2*$^{-/-}$ mice, and the chemoprotective activities of oltipraz and sulforaphane were lost (33–35). Similarly, *Nrf2*$^{-/-}$ mice are more susceptible to the acute toxicities of acetaminophen, diesel exhaust, 2,3-butyl-4-hydroxytoluene, and hyperoxia (36–40). These results argue that the Nrf2-mediated induction of phase 2 and antioxidant enzymes is critical for cellular defense against electrophilic and oxidative stresses. The results further suggest that oltipraz prevents

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chemical carcinogenesis by inducing Nrf2-regulated cytoprotective enzymes.

The contribution of the Nrf2 regulatory pathway in protection against urinary bladder carcinogenesis requires clarification, even though a large number of studies on chemically induced cancer formation have been reported. Thus, we investigated the susceptibility of *Nrf2*$^{-/-}$ mice to the urinary bladder-specific carcinogen BBN and the preventive efficacy of oltipraz in these mice. In wild-type mice, oltipraz up-regulated the detoxification activity of carcinogens in the liver and consequently decreased the BCPN concentration in the urine. Importantly, oltipraz also induced the expression of phase 2 enzyme genes in the wild-type urinary bladder and counteracted BBN-induced suppression of *UGT1A* gene expression. In *Nrf2*$^{-/-}$ mice, loss of Nrf2 significantly enhanced susceptibility to BBN and largely abolished the chemopreventive efficacy of oltipraz. These results show that the cellular defense enzymes under the regulation of Nrf2 play key roles in preventing urinary bladder carcinogenesis.

MATERIALS AND METHODS

Reagents. BBN was purchased from Tokyo Kasei (Tokyo, Japan). The Chemoprevention Branch of the National Cancer Institute (Bethesda, MD) provided the oltipraz. UDP-glucuronic acid (UDPGA) was purchased from Sigma (St. Louis, MO). Dr. Yukio Mori (The Gifu Pharmaceutical University, Gifu, Japan) provided the BCPN.

Animals. Nrf2-deficient mice of ICR/129SV background have been established at the University of Tsukuba (Tsukuba, Ibaraki, Japan; ref. 30). A colony of ICR/129SV background mice were backcrossed for nine generations with C57BL/6 mice, which were purchased from CLEA Japan (Tokyo, Japan). Mice were housed in stainless steel cages in an animal room maintained at 24 ± 2 °C. Mice were maintained with a 12-hour light/dark cycle and fed a purified AIN-76A diet (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*.

BBN-Induced Bladder Carcinogenesis. Oltipraz was fed *ad libitum* at the concentration of 250 mg/kg diet from 1 week before carcinogen administration until termination of the study 18 weeks later. BBN was dissolved in tap water to a concentration of 0.05% and supplied *ad libitum* for 8 weeks with the dark bottles. After the experimental period, mice were analyzed by autopsy. Urinary bladders were removed and inflated with and fixed in 10% buffered formalin. Each bladder then was sectioned sagittally, and each cup-shaped area was cut into four pieces. These eight strips of bladder tissue were serially embedded in one block of paraffin, cut into thin sections, and stained with H&E. Bladder lesions were histologically diagnosed according to the criteria of Oyasu *et al.* (41).

RNA Blot Analysis. Total RNAs from liver and whole urinary bladder were extracted with Isogen (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total RNAs (10 μg) were separated by 1.5% agarose gel electrophoresis containing 2.2 mol/L formaldehyde and transferred to nylon membrane. DNA probes for Nrf2 and UGT1A6 have been described previously (37), and Dr. Kimihiko Satoh (Hirosaki University School of Medicine, Hirosaki, Japan) provided probe for GSTπ (GSTP). DNA probes for UGT1A7 and total UGT1A were prepared by PCR using the following sets of primers: UGT1A7 sense primer, 5′-GCAGATGGTTGTGGAGAAACTC-3′; with antisense primer, 5′-GAGGTCTGTCATAGTCACTGG-3′; total UGT1A sense primer, 5′-AGCCTATGTCAACGCCTCTGG-3′; and with antisense primer, 5′-CCACTTCTCAATGGGTCTTGG-3′.

Establishment of Primary Cultures of Mouse Urothelial Cells. We adopted and modified the protocol to isolate bladder epithelium from male mice (42). Briefly, after the whole bladder was excised, it was everted to expose the mucosal surface. The bladder was digested in 20 units of dispase (Life Technologies, Inc., Rockville, MD) in PBS for 1 hour at 37°C. Following digestion, the bladder mucosa was gently detached from the underlying muscle tissue using fine-toothed forceps with coarse tips under a dissecting microscope. Mucosa was collected in PBS and further digested with 0.15% trypsin/EDTA at 37°C for 5 to 10 minutes. Trypsinized cells were mechanically dissociated by rigorous pipetting, filtered through a 100-μm nylon cloth, and centrifuged at 200 × *g* for 5 minutes. Approximately 5 to 10 × 10^5 cells were seeded in a 50-mm plastic dish containing a 1:1 mixture of serum-free keratinocyte medium and DMEM with 5% (v/v) fetal bovine serum, epidermal growth factor (5 ng/mL), bovine pituitary extract (50 μg/mL), cholera toxin (30 ng/mL), penicillin (100 units/mL), and streptomycin (1 μg/mL). The reagents used for this culture experiment were from Life Technologies.

Immunoblot Analysis. The nuclei of mouse hepatic cells and primary mouse urothelial cells prepared as described previously were solubilized with SDS-sample buffer without loading dye and 2-mercaptoethanol. Protein concentrations were estimated by BCA protein assay (Pierce, Rockford, IL). Proteins were separated by 6.0% SDS-PAGE and electrotransferred onto an Immobilon membrane (Millipore, Bedford, MA). Anti-Nrf2 antibody was used as described previously (32). Drs. Shigeru Taketani (Kyoto Institute of Technology, Kyoto, Japan) and John Hayes (University of Dundee, Dundee, United Kingdom) provided anti-heme oxygenase 1 (HO-1) and anti-GSTA1/A2 antibodies, respectively. Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-IgG antibody and enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Determination of BCPN. The urinary level of BCPN was determined as reported previously (43) with modification. The urine sample (0.1 mL) was diluted to 0.5 mL with distilled water before assay. A 3.3-μL aliquot of 12 mol/L HCl was added, and the sample was extracted with 0.5 mL of ethyl acetate three times. The organic layers were collected after centrifugation for 5 minutes at 10,000 × *g* and dried using a speed vacuum concentrator with a cooling trap <30°C. The residues dissolved in ethyl acetate were spotted onto a silica gel 70 F_{254} precoated plate (Wako, Osaka, Japan) and developed with chloroform/methanol/acetic acid (18:1:1, v/v) in the dark. The bands corresponding to BBN or BCPN (Rf = 0.68 to 0.72) were scraped off and eluted from the silica with 4 mL acetone. The eluates then were concentrated by speed vacuum as before and diluted with acetonitrile to a final volume of 0.2 mL. Samples were filtered through a MINISART RC4 filter (0.2-μm pore size; Sartorius, Gottingen, Germany) and analyzed by high-performance liquid chromatography (HPLC). The urinary BCPN level was determined with a Shimazu LC9A apparatus (Shimazu, Kyoto, Japan) on a Finepak SIL C_{18} column (Jasco, Tokyo, Japan; 250 × 4.6 mm, inner diameter) at 239 nm. Separation was performed with a mobile phase consisting of a 3:7 mixture

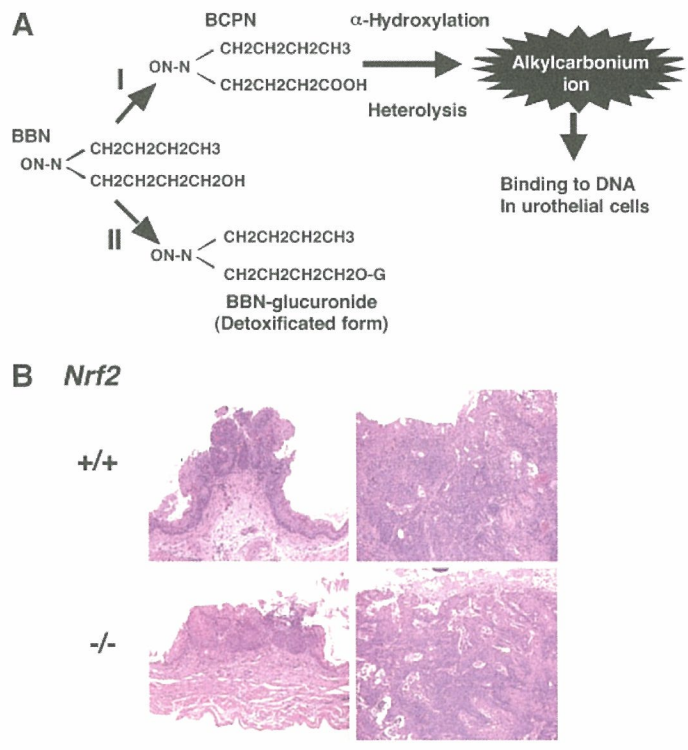

Fig. 1. BBN-induced carcinogenesis in wild-type and *Nrf2*$^{-/-}$ mouse urinary bladders. *A*, biotransformation processes of BBN; *G*, glucuronic acid. *B*, histopathologic analysis of tumor regions. Tissue sections of urinary bladder from wild-type (*top*) and *Nrf2*$^{-/-}$ (*bottom*) mice were analyzed by H&E staining. Noninvasive carcinoma (*left*) and invasive carcinoma (*right*) are shown.

Table 1 *BBN-induced carcinogenesis of the urinary bladder in wild-type and Nrf2$^{-/-}$ male mice and effect of oltipraz on the carcinogenesis*

Genotype	Oltipraz treatment	Cancer incidence Number (%)	Invasive cancer incidence Number (%)	Total number (entry number)
Wild type	−	9 (36.0)	6 (24.0)	25 (26)
	+	4 (13.8)	1 (3.4)*	29 (29)
Nrf2−/−	−	17 (65.4)*	10 (38.5)	26 (27)
	+	15 (65.2)*	6 (26.1)	22 (26)

* $P < 0.05$ compared with untreated wild-type mice.

(v/v) of acetonitrile and 20 mmol/L sodium acetate buffer (pH 4.5) at a flow rate of 1 mL/min. Under these conditions, the retention time of BCPN was 7.8 minutes. The recovery rate of BCPN from the urine was ~60% in our assay conditions.

Measurement of BBN-Glucuronide *In vitro*. Microsomes were prepared from mouse liver as described previously (44). A typical reaction mixture consisted of 100 mmol/L potassium phosphate buffer (pH 7.4), 1 mmol/L BBN, 5 mmol/L UDPGA, 0.05% Brij 58, and microsome preparation (600 μg) in a final volume of 1.0 mL. Reactions were initiated by the addition of BBN, and incubations were performed at 37°C for 30 minutes. BSA (1 mg) and 24% trichloroacetic acid (0.1 mL) were added to the incubation mixture to terminate the reaction. After centrifugation at 10,000 × *g* for 5 minutes, the supernatant (0.1 mL) was injected into the HPLC as described previously. Separation of BBN and its glucuronide was carried out with a mobile phase consisting of a 2:8 mixture (v/v) of acetonitrile and 20 mmol/L sodium acetate buffer (pH 4.5) at a flow rate of 1 mL/min.

Statistical Analyses. Data were expressed as mean ± SEM. The Student *t* test was used to determine the statistical difference among groups. The values for urinary bladder incidence were analyzed using the χ^2 or Fisher's exact probability test. A *P* value < 0.05 was accepted as statistically significant.

RESULTS

High Susceptibility of *Nrf2*$^{-/-}$ Mice to BBN-Induced Carcinogenesis. BBN is metabolized primarily through two pathways (45): one is alcohol/aldehyde dehydrogenase-mediated oxidation to yield BCPN, whereas the other is UGT-catalyzed conjugation to form BBN-glucuronide (Fig. 1; pathways I and II, respectively). Because glucuronide conjugation is an important process for detoxifying reactive chemicals, it has been suggested that a change in the distribution of BBN metabolites, such as a decrease in BCPN or an increase in BBN-glucuronide, might affect the incidence of tumor formation during exposure to BBN.

To elucidate the roles of Nrf2 in the prevention of urinary bladder carcinogenesis by BBN, we examined the susceptibility of *Nrf2*$^{-/-}$ mice to BBN carcinogenesis. Although *Nrf2*$^{-/-}$ mice were slightly heavier (<2.0 g) than wild-type animals, there was no significant difference in the body weight gained between the two groups during the experimental period. Several mice died before the end of the experiment (Table 1). In the group of wild-type mice, one mouse died within the experimental period, and its death was not attributable to BBN treatment. Conversely, five mice from the group of *Nrf2*$^{-/-}$ died before the end of the experiment. Autopsy revealed abdominal masses

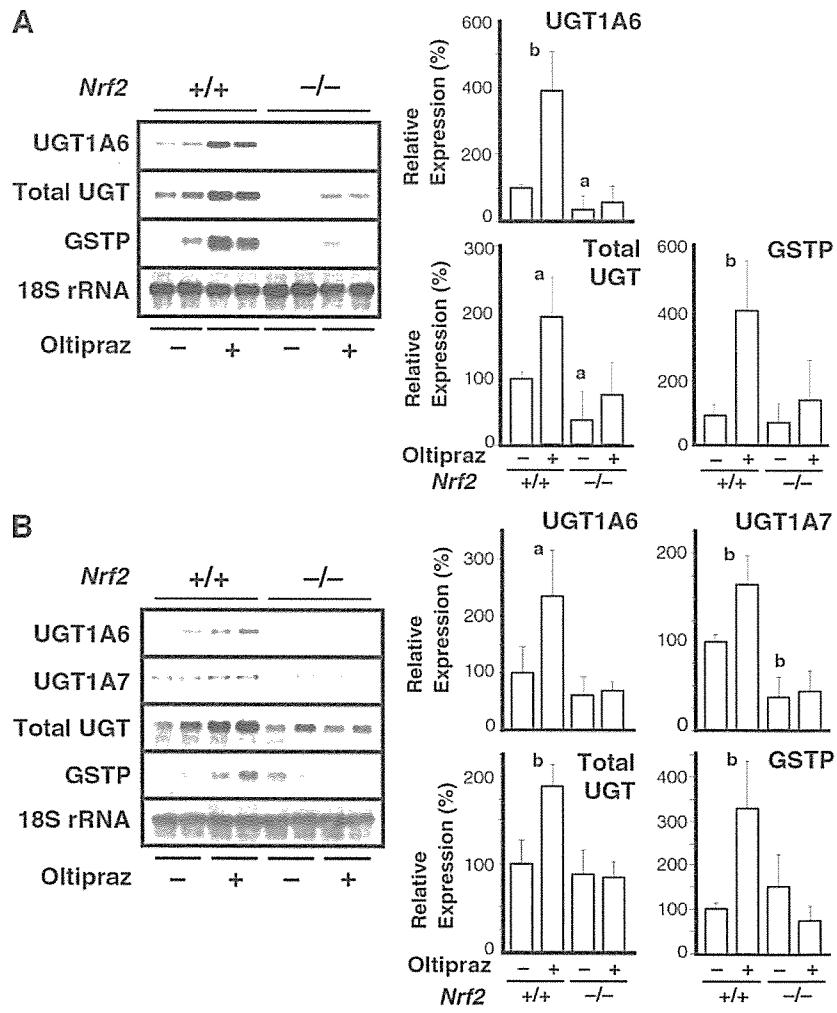

Fig. 2. Effect of oltipraz on the expression of phase 2 enzyme mRNAs in the liver and urinary bladder. *A* and *B*, effect of oltipraz on the expression of phase 2 enzyme genes in the liver (*A*) and urinary bladder (*B*) in wild-type and *Nrf2*$^{-/-}$ male mice. Oltipraz was fed at the concentration of 1 g/kg diet for 48 hours. Densitometric data of RNA blot analysis were normalized by 18S rRNA and expressed as ratios to vehicle-treated controls. Values are represented as mean ± SE ($n = 4$). *a*, $P \leq 0.05$ compared with nontreated wild-type mice. *b*, $P \leq 0.01$ compared with nontreated wild-type mice.

involving kidney and lymph nodes in three of these dead mice, apparently attributable to the BBN treatment. Bladder lesions were diagnosed histologically according to the previously described criteria (41). All of the noninvasive carcinomas were nodular rather than papillary in shape. The term "cancer" has been applied to transitional and squamous cell carcinomas because most of the lesions contained both components. No pathologic differences in noninvasive (Fig. 1*B, top* and *bottom left*) and invasive tumors (Fig. 1*B, top* and *bottom right*) were found between wild-type and *Nrf2*$^{-/-}$ mice, respectively.

Table 1 summarizes the incidence of urinary bladder cancer caused by BBN treatment. The incidence of noninvasive and invasive carcinoma was significantly higher in *Nrf2*$^{-/-}$ mice (65.4%) than in wild-type mice (36.0%; $P = 0.036$). In BBN-treated mice, invasive carcinoma was found in 38.5% and 24.0% of *Nrf2*$^{-/-}$ and wild-type mice, respectively. In wild-type mice, oltipraz treatment reduced the incidence of urinary bladder cancer by 61.6% and the incidence of invasive cancer by 85% ($P = 0.041$). However, in *Nrf2*$^{-/-}$ mice, oltipraz significantly lost its chemopreventive efficacy, although oltipraz partially reduced the incidence of invasive cancer. These results clearly indicate that detoxifying enzymes under Nrf2 regulation contribute to the cancer chemopreventive effect of oltipraz.

Expression of Phase 2 Genes in the Liver and Urinary Bladder of *Nrf2*$^{-/-}$ and Wild-Type Mice Treated with Oltipraz. To elucidate the roles that Nrf2 may play in the protection against BBN carcinogenesis afforded by oltipraz, we examined changes in the expression of detoxifying enzyme genes in the liver and urinary bladder following oltipraz treatment. For this purpose, oltipraz (1 g/kg) was added to the diet and fed to mice for 48 hours. The mRNA levels of UGT1A6, total UGT1A, and GSTP were monitored by RNA blot analysis. The constitutive expression of these detoxifying genes was 40 to 50% lower in the livers of *Nrf2*$^{-/-}$ mice than in wild-type mice (Fig. 2*A*). Although oltipraz increased the mRNA levels of UGT1A6 and GSTP by approximately fourfold and that of total UGT1A by twofold in the livers of wild-type mice, the inducible expression of these genes by oltipraz was markedly reduced in the livers of *Nrf2*$^{-/-}$ mouse (Fig. 2*A*).

Next, the expression profiles of these detoxifying enzyme genes in the urinary bladder were examined. We found that the basal level of these detoxifying enzyme mRNAs in the bladder were lower in *Nrf2*$^{-/-}$ mice than in wild-type mice (Fig. 2*B*). Oltipraz induced the expression of these enzymes in the urinary bladder of wild-type mice, but the magnitude of induction was less, approximately twofold for UGT1A6 and threefold for GSTP. The inducible expression of these genes by oltipraz was significantly abrogated in the *Nrf2*$^{-/-}$ mouse urinary bladder (Fig. 2*B*). We also examined the expression of UGT1A7 mRNA. UGT1A7 mRNA was detected in the urinary bladder (Fig. 2*B*) but not in the liver (data not shown), and the constitutive and inducible expressions were affected in the *Nrf2*$^{-/-}$ mouse urinary bladder. These results revealed that phase 2 detoxifying enzymes are expressed in the urinary bladder and that Nrf2 regulates their expression in response to electrophilic inducers.

Nrf2 Regulatory Pathway Is Activated in Liver and Urothelial Cells. We next examined Nrf2 activation by oltipraz in liver and urothelial cells. The mRNA levels of Nrf2 itself did not change substantially on treatment with oltipraz in either tissue (Fig. 3*A*). Because we carried out a targeting knockout of the *Nrf2* gene by introducing the β-galactosidase gene into the *Nrf2* locus, creating Nrf2–β-galactosidase fusion mRNA (30), we detected larger-sized mRNA in the *Nrf2*$^{-/-}$ mice. The level of the larger-sized mRNA did not change much on treatment with oltipraz (Fig. 3*A*). These observations are consistent with our contention that activation of Nrf2 correlates with nuclear accumulation of Nrf2 protein. To confirm this point further, we examined the nuclear expression of Nrf2 protein in the liver after treatment with oltipraz. Immunoblot analysis showed an increased nuclear accumulation of Nrf2 protein in wild-type mice, but not in *Nrf2*$^{-/-}$ mice, following exposure to oltipraz (Fig. 3*B*).

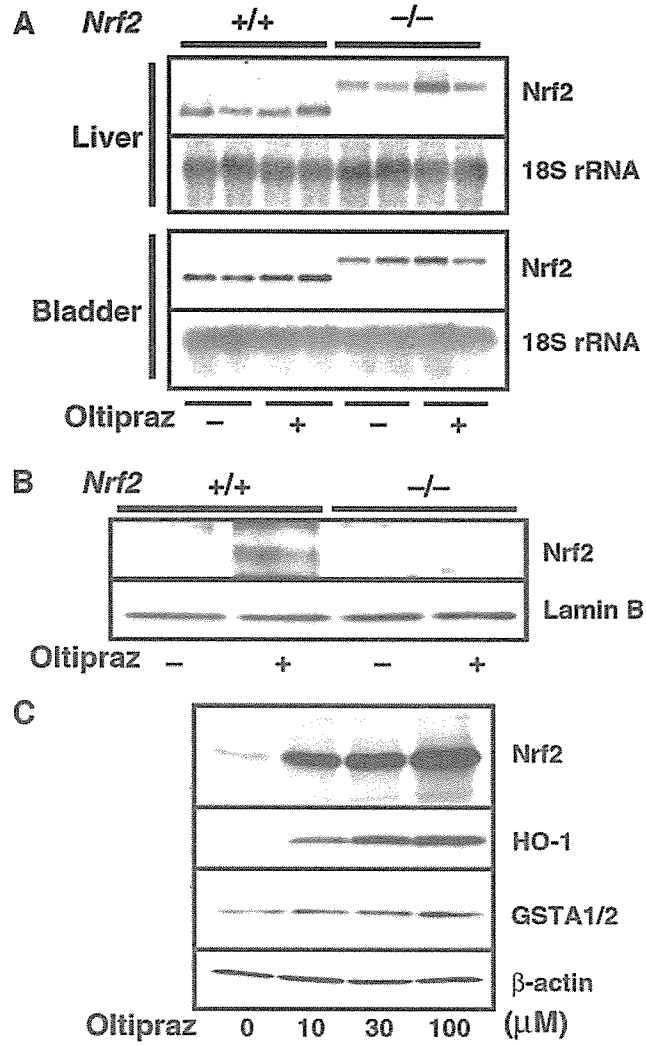

Fig. 3. Effect of oltipraz on Nrf2 activation in the liver and urinary bladder. *A*, effect of oltipraz on the expression of Nrf2 mRNA in the liver and urinary bladder of male wild-type and *Nrf2*$^{-/-}$ mice. Mice were fed oltipraz at the concentration of 1 g/kg diet for 48 hours. Densitometric analysis of RNA blot results was normalized by 18S rRNA levels and expressed as ratios to vehicle-treated controls. Values are represented as mean ± SE ($n = 4$). *B*, Nrf2 activation in mouse liver by oltipraz. Male wild-type and *Nrf2*$^{-/-}$ mice were fed oltipraz at the concentration of 1 g/kg diet for 48 hours, and hepatic nuclear extracts were examined by immunoblot analysis using anti-Nrf2 antibody. Lamin B was used as a loading control. *C*, immunoblot analyses of Nrf2, HO-1, and GSTA1/2 in mouse uroepithelial primary cell cultures. Total cell extract prepared from wild-type uroepithelial cells was treated with 10, 30, and 100 μmol/L oltipraz or vehicle for 8 hours. β-Actin was used as a loading control.

To date, there have been no reports describing the expression of Nrf2 and its target genes in urothelial cells. However, the Nrf2-dependent expression of phase 2 enzyme genes in the urinary bladder suggests that the Nrf2 regulatory pathway is functioning in urothelial cells. We clarified that this is the case by establishing a primary urothelial cell culture system and examining the expression of Nrf2. Immunoblot analysis of total cell extracts with anti-Nrf2 antibody showed that the amount of Nrf2 protein is increased by oltipraz in a dose-dependent manner (Fig. 3*C*). Oltipraz also induced the nuclear accumulation of Nrf2 (data not shown) and the expressions of HO-1 and GSTA1/A2 (Fig. 3*C*).

Elevated BCPN Concentration in the Urine of *Nrf2*$^{-/-}$ Mice. BCPN is a proximate metabolite of BBN, and BCPN and BBN are metabolized through α-hydroxylation/spontaneous cleavage to pro-

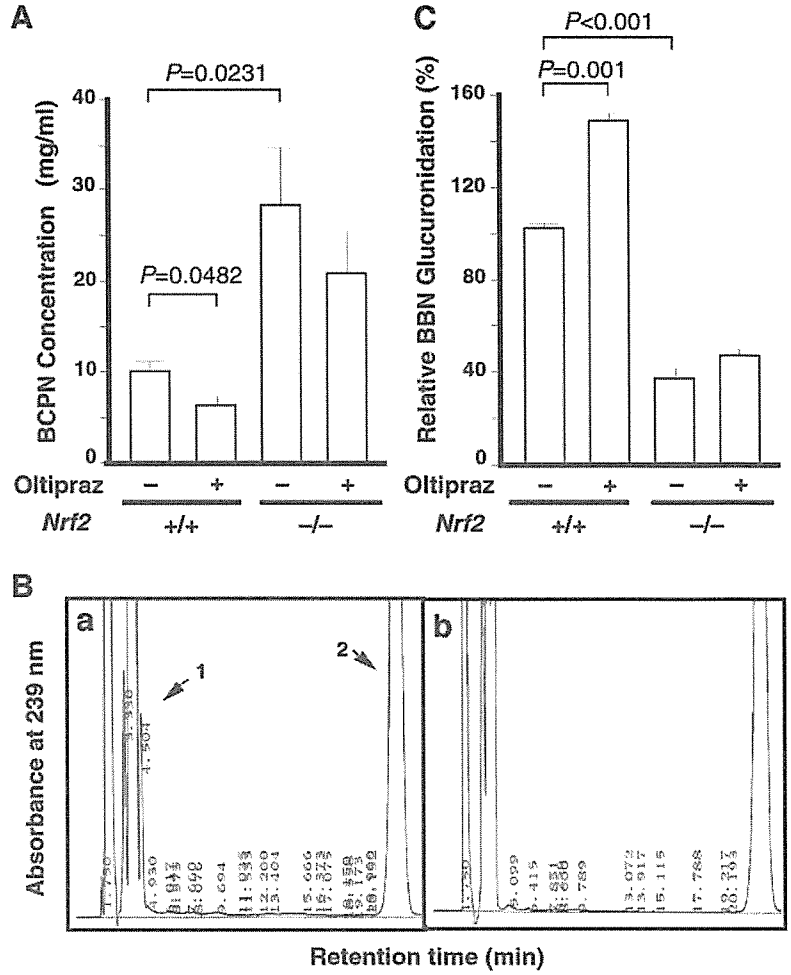

Fig. 4. Effect of *Nrf2* genotype and oltipraz treatment on the urinary concentration of BCPN after treatment with BBN and the activity of BBN glucuronidation in hepatic microsomes. *A*, Mice were fed 250 mg/kg diet of oltipraz and 0.05% BBN in drinking water for 2 weeks, and then urine samples were analyzed by HPLC. Values are represented as mean ± SE ($n = 5$). *Significantly different from nontreated wild-type mice ($P \leq 0.05$). *B*, Mice given oltipraz at the concentration of 250 mg/kg diet for 2 weeks were used. Hepatic microsomes from the animals were prepared as described in Materials and Methods. Six hundred micrograms of Brij 58-solubilized microsomes from mouse livers were incubated with 1 mmol/L BBN in the presence of 5 mmol/L UDPGA at 37°C for 30 minutes. *a*, complete system; *b*, without enzyme preparation. Peaks 1 and 2 were identified as BBN-glucuronide and BBN, respectively. *C*, The relative formation of BBN-glucuronide by liver microsomes from mice treated either with or without oltipraz treatment. Values are represented as mean ± SE ($n = 3$). *Significantly different from untreated wild-type mice ($P \leq 0.05$).

duce their alkylcarbonium ion. These reactive species can covalently bind to DNA and are associated with the formation of a butyl-guanine adduct in the urothelial DNA of animals treated with BBN (16). We hypothesized that increased carcinogenesis in $Nrf2^{-/-}$ mice is associated with a higher than normal urinary BCPN concentration. We measured by HPLC the urinary concentration of BCPN 2 weeks after administration of 0.05% BBN to mice treated either with or without oltipraz (Fig. 4*A*). Mice were fed oltipraz (250 mg/kg) 1 week before BBN administration. The urinary concentration of BCPN was significantly higher in $Nrf2^{-/-}$ mice than in wild-type mice ($P = 0.0231$). Oltipraz treatment significantly reduced the urinary concentration of BCPN in wild-type mice ($P = 0.0482$) but not in $Nrf2^{-/-}$ mice.

Oltipraz Enhanced BBN Glucuronidation Activity in Liver Microsomes. Considering that BBN glucuronidation occurs mainly in the liver, it is reasonable to assume that an increase in BBN glucuronidation in the liver would contribute, at least in part, to a decrease in BCPN concentration in the urine and consequent suppression of carcinogenesis in the urinary bladder. Therefore, we measured the glucuronidation activity of BBN in hepatic microsomes *in vitro* by HPLC. Incubation of BBN (peak 2) with the Brij 58-solubilized microsome of wild-type mouse liver in the presence of UDPGA resulted in a new product (peak 1) with a retention time of 4.5 minutes (Fig. 4*B*, *a*). This metabolite was not detected when the enzyme preparation (Fig. 4*B*, *b*), UGPGA (data not shown), or BBN (data not shown) was excluded from the incubation mixture, indicating that the product was BBN-glucuronide generated from BBN.

The basal activity of BBN glucuronidation was significantly lower in the hepatic microsomes of $Nrf2^{-/-}$ mice than in wild-type mice ($P = 0.001$). Oltipraz significantly induced the BBN glucuronidation activity in wild-type mouse liver microsomes ($P = 0.001$) but not in $Nrf2^{-/-}$ mouse liver microsomes (Fig. 4*C*). Collectively, these results suggest that the administration of oltipraz reduces the concentration of BCPN in the urine by enhancing the hepatic BBN glucuronidation activity.

BBN Decreases UGT Expression, and Oltipraz Counteracts the Suppression in Urinary Bladder. It was reported previously that *UGT1A* gene expression in cancerous human urinary bladder was either lost or decreased to a low level compared with that in normal bladder tissue (13). Such down-regulation of UGT expression in the urinary bladder may reduce the local glucuronidation activity of carcinogenic compounds, allowing their accumulation and promoting DNA mutations in the urinary bladder.

We analyzed the effect of BBN on *UGT1A* gene expression in the urinary bladder by supplementing drinking water with 0.01%, 0.05%, or 0.1% BBN for 2 weeks. The expressions of *UGT1A6*, *UGT1A7*, and total *UGT1A* were significantly decreased by BBN treatment in a dose-dependent manner (Fig. 5*A*). Importantly, this pattern of *UGT1A* suppression by BBN also was observed in $Nrf2^{-/-}$ mice (Fig. 5*B*). We tested whether oltipraz counteracts the down-regulation of *UGT1A* gene expression by BBN. Mice were given 250 mg/kg of oltipraz in the diet 1 week before carcinogen administration (0.01% BBN) in the drinking water for 2 weeks. In wild-type mice, BBN decreased the expressions of *UGT1A6*, *UGT1A7*, and total *UGT1A* by 50.1%, 54.0%, and 52.0%, respectively, whereas in $Nrf2^{-/-}$ mice, BBN markedly reduced the expressions of *UGT1A6*, *UGT1A7*, and total *UGT1A* to <10% (Fig. 5*A* and *C*). Oltipraz effectively inhibited

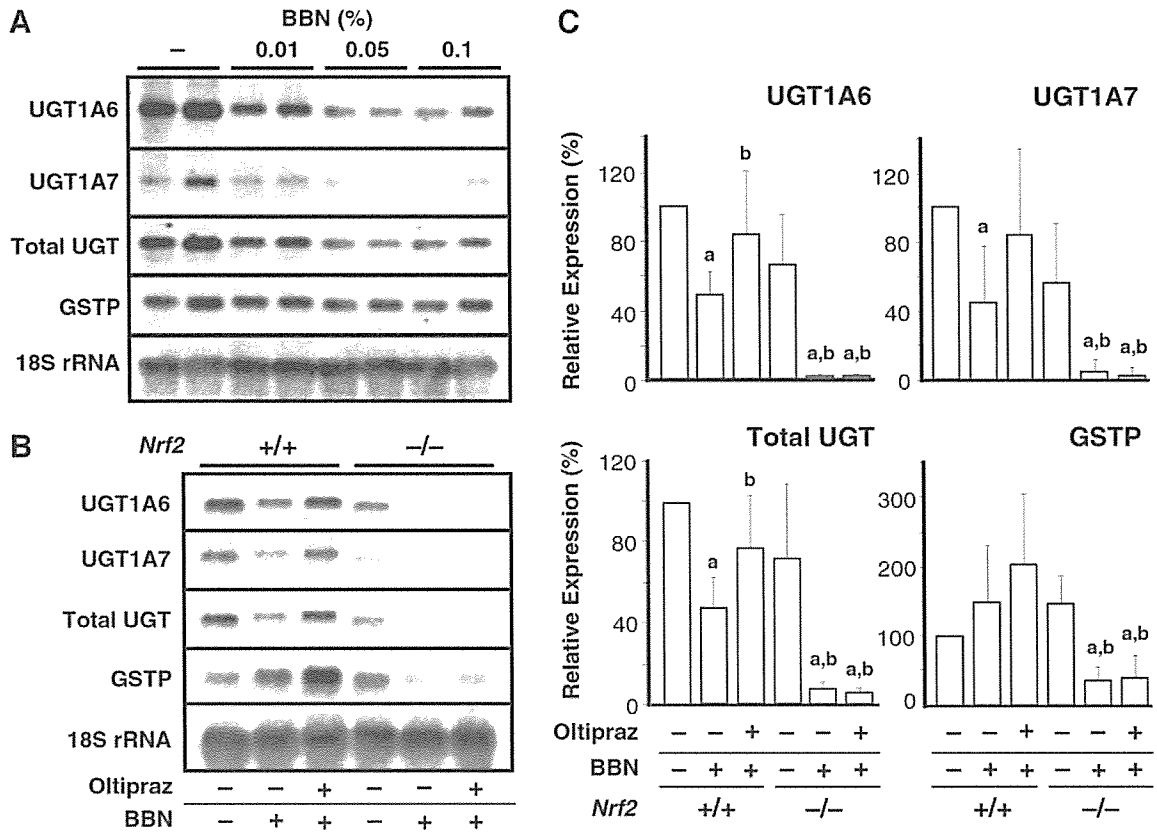

Fig. 5. Effect of BBN and oltipraz on the expressions of phase 2 genes. *A*, effect of BBN on the expressions of phase 2 genes in the urinary bladder of male wild-type mice. Mice were treated with 0.01%, 0.05%, 0.1% BBN, or vehicle in drinking water for 2 weeks. *B*, The expressions of phase 2 genes after BBN and oltipraz treatment were examined in wild-type and *Nrf2*$^{-/-}$ mice. Oltipraz was fed at the concentration of 250 mg/kg diet 1 week before carcinogen administration. BBN was given at a concentration of 0.01% in drinking water for 2 weeks. *C*, Densitometric analysis of RNA blot results was normalized by 18S rRNA levels and expressed as ratios to vehicle-treated controls. Values are represented as mean ± SE ($n = 4$). *a*, $P \leq 0.05$ compared with nontreated wild-type mice. *b*, $P \leq 0.05$ compared with BBN-treated wild-type mice.

the down-regulation of *UGT1A* genes caused by BBN in the urinary bladder of wild-type mice but completely lost its efficacy in *Nrf2*$^{-/-}$ mice (Fig. 5*B* and *C*). BBN did not suppress *GSTP* gene expression, indicating that BBN specifically targets *UGT1A* genes (Fig. 5). Thus, these results show that BBN suppresses *UGT1A* gene expression in the urinary bladder through mechanisms independent of the Nrf2 regulatory pathway.

DISCUSSION

Our study has shown that *Nrf2*$^{-/-}$ mice are more susceptible to BBN-induced carcinogenesis of the urinary bladder than wild-type mice. The elevated incidence of BBN carcinogenesis in *Nrf2*$^{-/-}$ mice was associated with the higher concentration of BCPN in the urine and lower activity of BBN-glucuronidation in the liver. Whereas oltipraz effectively reduced the incidence of urinary bladder carcinoma initiated by BBN in wild-type C57BL/6 mice, it showed little effect in *Nrf2*$^{-/-}$ mice. In wild-type mice, oltipraz significantly increased the activity of BBN-glucuronidation in the liver, an increase that correlated well with the increased *UGT1A* gene expression, and thereby reduced the urinary concentration of BCPN. Furthermore, oltipraz increased the expression of phase 2 enzyme genes and suppressed the BBN-induced down-regulation of *UGT1A* expression in urinary bladder in an Nrf2-dependent manner. Collectively, these results highlight the importance of a set of detoxifying and cytoprotective enzymes under the regulatory influence of Nrf2 in the prevention of urothelial carcinogenesis.

Epidemiologic and experimental lines of evidence also suggest that the activity of detoxifying enzymes is tightly linked to urinary bladder carcinogenesis. However, the mechanism as to how the decrease in detoxifying enzyme activity contributes to carcinogenesis of the urinary bladder remains to be clarified. It was reported previously that oltipraz, an inducer of phase 2 detoxifying enzymes, reduces the incidence of bladder cancer caused by BBN (46). Exploiting *Nrf2*$^{-/-}$ mice for the BBN-carcinogenesis experiment, this study proved that oltipraz acts to prevent the initiation of cancer through activation of detoxification enzymes under Nrf2 regulation. It is of note that oltipraz repressed the incidence of invasive cancer and urinary BCPN concentration even in *Nrf2*$^{-/-}$ mice, indicating that oltipraz exerts its chemopreventive function partially through a pathway independent of Nrf2. Oltipraz was reported to induce *GSTA2* gene expression by activating CAAT/enhancer binding protein β (47).

One salient observation in this study was that the detoxification processes in the liver and urinary bladder act simultaneously and cooperatively to prevent chemical carcinogenesis of the urinary bladder. Our current model for the roles of Nrf2 and its downstream gene products in protection against BBN carcinogenesis is summarized in Fig. 6. In this model, oltipraz prevents BBN carcinogenesis primarily through the induction of BBN glucuronidation in the liver. Oltipraz also induces phase 2 and antioxidant enzymes in the urinary bladder in an Nrf2-dependent manner. Because BBN and BCPN are metabolized to reactive species in urothelial cells, it is likely that the defense system in the urinary bladder plays a key role in the anticarcinogenic mechanism (16). Therefore, induction of Nrf2-mediated detoxifying enzymes in the peripheral urothelial cells and in liver may become an important strategy to prevent BBN-induced bladder carcinogenesis.

It has been shown that decreased expression of phase 2 detoxifying

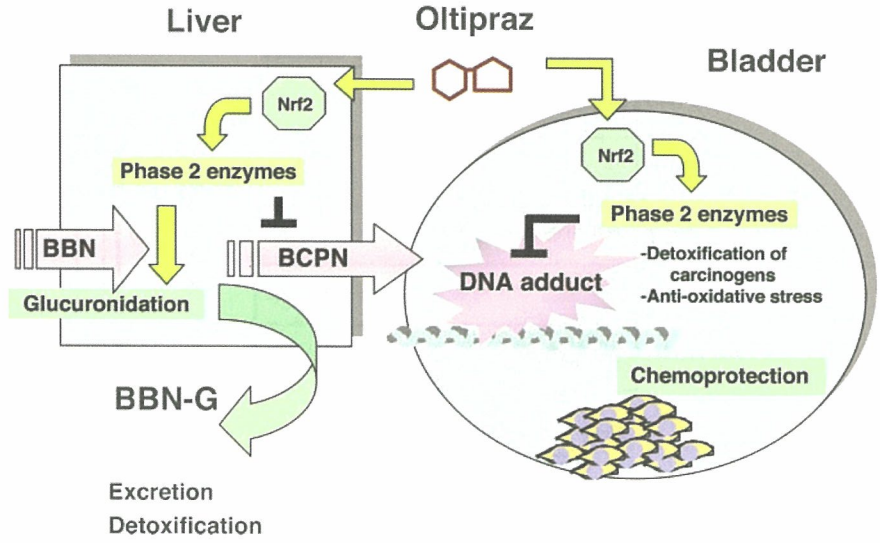

Fig. 6. Mechanism of chemoprotection by oltipraz against urinary bladder carcinogenesis. Oltipraz reduced BBN-induced carcinogenesis by suppressing the urinary excretion of BCPN by means of Nrf2-dependent induction of BBN glucuronidation in the liver. Moreover, oltipraz also works in the urinary bladder by inducing phase 2 enzymes and antioxidant proteins, such as HO-1, to suppress BBN-induced carcinogenesis. Furthermore, oltipraz counteracted the BBN-provoked urinary bladder-specific suppression of *UGT1A* gene expression in an Nrf2-dependent manner.

enzymes predisposes cells to neoplastic transformation. For example, Nelson *et al.* (48) reported that the loss of *GSTP1* expression in the prostate precedes neoplastic transformation. Expression of the *GSTP1* gene, which is the major GST isoform expressed in normal human prostate, is silenced in the majority of prostate tumors by the hypermethylation of CpG islands residing in the 5′ regulatory region. Conversely, overexpression of *GSTP1* in the prostate cell line LNCaP inhibited the cytotoxicity and DNA-adduct formation caused by a potential dietary carcinogen (49). Down-regulation of *UGT1A* gene expression also was found in an early stage of hepatocarcinogenesis (50).

In the case of urinary bladder cancer, it has been reported that carcinogenesis is associated with a decrease in or loss of *UGT1A* gene expression (13). Therefore, the finding that BBN acts to repress *UGT1A* gene expression in a urinary bladder-specific manner is intriguing. We found in this study that BBN significantly decreases *UGT1A* gene expression in a dose-dependent manner and that this decrease is observed as early as 1 day after administration of BBN (data not shown). This down-regulation of *UGT1A* leads to increased BBN or BCPN levels in urothelial cells, which may ultimately increase DNA alkylation. These observations also suggest the presence of bladder-specific regulation of *UGT1A* gene expression, which is sensitive to BBN. Because suppression also was observed in $Nrf2^{-/-}$ mice, the mechanism seems to be independent of Nrf2 regulation. In contrast, oltipraz counteracted the BBN-induced suppression in an Nrf2-dependent manner, suggesting that expression of *UGT1A* genes is under multiple regulatory influences. The Nrf2 regulatory pathway may compensate for the BBN-induced down-regulation of *UGT1A* gene expression in wild-type mice.

It was reported that *p53* gene knockout mice ($p53^{+/-}$ mice) are susceptible to BBN-induced urinary bladder carcinogenesis (43). The high susceptibility of $p53^{+/-}$ mice to BBN was associated with an increased cell proliferation without alteration of BCPN concentration in the urine. If we consider the high level of BCPN in the urine of $Nrf2^{-/-}$ mice, the mechanism that makes $Nrf2^{-/-}$ mice susceptible to BBN carcinogenesis must be different from that observed in $p53^{+/-}$ mice. Therefore, the use of a combination of oltipraz and other chemopreventive agents with distinct molecular targets would provide a strong synergistic efficacy. An attractive prospect also would be the discovery of more powerful chemical agents that are specifically delivered to the urinary bladder to induce the expression of phase 2 enzyme genes. Such strategies may be of importance in the protection against urinary bladder carcinogenesis.

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REFERENCES

1. Wai CY, Miller DS. Urinary bladder cancer. Clin Obstet Gynecol 2002;45:844–54.
2. Case RA, Hosker ME, McDonald DB, Pearson JT. Tumours of the urinary bladder in workmen engaged in the manufacture and use of certain dyestuff intermediates in the British chemical industry. Part I. The role of aniline, benzidine, α-naphthylamine, and β-naphthylamine. Br J Ind Med 1954;11:75–96.
3. Tricker AR, Mostafa MH, Spiegelhalder B, Preussmann R. Urinary excretion of nitrate, nitrite and N-nitroso compounds in schistosomiasis and bilharzia bladder cancer patients. Carcinogenesis 1989;10:547–52.
4. Mirvish SS. Role of N-nitroso compounds (NOC) and N-nitrosation in etiology of gastric, esophageal, nasopharyngeal and bladder cancer and contribution to cancer of known exposures to NOC. Cancer Lett 1995;93:17–48.
5. Druckrey H, Preussmann R, Ivankovic S, Schmahl D. Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats. Z Krebsforsch 1967;69:103–201.
6. IARC. N-Nitrosodi-n-butylamine. IARC Monogr Eval Carcinog Risk Chem Man 1978;17:51–75.
7. Okada M, Ishidate M. Metabolic fate of N-n-butyl-N-(4-hydroxybutyl)-nitrosamine and its analogues. Selective induction of urinary bladder tumours in the rat. Xenobiotica 1977;7:11–24.
8. Ito N, Hiasa Y, Tamai A, Okajima E, Kitamura H. Histogenesis of urinary bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine in rats. Gann 1969;60:401–10.
9. Brockmoller J, Cascorbi I, Kerb R, Roots I. Combined analysis of inherited polymorphisms in arylamine N-acetyltransferase 2, glutathione S-transferases M1 and T1, microsomal epoxide hydrolase, and cytochrome P450 enzymes as modulators of bladder cancer risk. Cancer Res 1996;56:3915–25.
10. Johns LE, Houlston RS. N-acetyl transferase-2 and bladder cancer risk: a meta-analysis. Environ Mol Mutagen 2000;36:221–7.
11. Johns LE, Houlston RS. Glutathione S-transferase ì1 (GSTM1) status and bladder cancer risk: a meta-analysis. Mutagenesis 2000;15:399–404.
12. Park SJ, Zhao H, Spitz MR, Grossman HB, Wu X. An association between NQO1 genetic polymorphism and risk of bladder cancer. Mutat Res 2003;536:131–7.
13. Giuliani L, Gazzaniga P, Caporuscio F, Ciotti M, Frati L, Agliano AM. Can down-regulation of UDP-glucuronosyltransferases in the urinary bladder tissue impact the risk of chemical carcinogenesis? Int J Cancer 2001;91:141–3.
14. Hashimoto Y, Kitagawa HS. In vitro neoplastic transformation of epithelial cells of rat urinary bladder by nitrosamines. Nature 1974;252:497–99.
15. Airoldi L, Magagnotti C, Bonfanti M, Fanelli R. α-Oxidative metabolism of the bladder carcinogens N-nitrosobutyl(4-hydroxybutyl)amine and N-nitrosobutyl(3-carboxypropyl)-amine within the rat isolated bladder. Carcinogenesis 1990;11:1437–40.
16. Airoldi L, Magagnotti C, Bonfanti M, et al. Detection of O6-butyl- and O6-(4-hydroxybutyl)guanine in urothelial and hepatic DNA of rats given the bladder carcinogen N-nitrosobutyl(4-hydroxybutyl)amine. Carcinogenesis 1994;15:2297–301.
17. Talalay P. Chemoprotection against cancer by induction of phase 2 enzymes. Biofactors 2000;12:5–11.
18. Talalay P, Dinkova-Kostova AT, Holtzclaw WD. Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv Enzyme Regul 2003;43:121–34.
19. Benson AM, Batzinger RP, Ou SY, Bueding E, Cha YN, Talalay P. Elevation of hepatic glutathione S-transferase activities and protection against mutagenic metabolites of benzo(a)pyrene by dietary antioxidants. Cancer Res 1978;38:4486–95.

20. Ansher SS, Dolan P, Bueding E. Biochemical effects of dithiolethiones. Food Chem Toxicol 1986;24:405–15.
21. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 1992;89:2399–403.
22. Clapper ML. Chemopreventive activity of oltipraz. Pharmacol Ther 1998;78:17–27.
23. Kensler TW, Groopman JD, Sutter TR, Curphey TJ, Roebuck BD. Development of cancer chemopreventive agents: oltipraz as a paradigm. Chem Res Toxicol 1999;12: 113–26.
24. Primiano T, Sutter TR, Kensler TW. Antioxidant-inducible genes. Adv Pharmacol 1997;38:293–328.
25. Friling RS, Bensimon S, Daniel V. Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. Proc Natl Acad Sci USA 1990;87:6258–62.
26. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. J Biol Chem 1991;266:11632–9.
27. Itoh K, Igarashi K, Hayashi N, Nishizawa M, Yamamoto M. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small maf family proteins. Mol Cell Biol 1995;15:4184–93.
28. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the α-globin locus control region. Proc Natl Acad Sci USA 1994;91:9926–30.
29. Venugopal R, Jaiswal AK. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. Proc Natl Acad Sci USA 1996;93:14960–5.
30. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313–22.
31. Chan K, Lu R, Chang JC, Kan YW. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. Proc Natl Acad Sci USA 1996;93:13943–8.
32. Ishii T, Itoh K, Takahashi S, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 2000;275: 16023–9.
33. Ramos-Gomez M, Kwak MK, Dolan PM, et al. Sensitivity to carcinogenesis is increased and chemoprotective efficacy of enzyme inducers is lost in nrf2 transcription factor-deficient mice. Proc Natl Acad Sci USA 2001;98:3410–5.
34. Ramos-Gomez M, Dolan PM, Itoh K, Yamamoto M, Kensler TW. Interactive effects of nrf2 genotype and oltipraz on benzo[a]pyrene-DNA adducts and tumor yield in mice. Carcinogenesis 2003;24:461–7.
35. Fahey JW, Haristoy X, Dolan PM, et al. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo-[a]pyrene-induced stomach tumors. Proc Natl Acad Sci USA 2002;99:7610–5.
36. Chan K, Han XD, Kan YW. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci USA 2001;98:4611–6.
37. Enomoto A, Itoh K, Nagayoshi E, et al. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicol Sci 2001;59: 169–77.
38. Chan K, Kan YW. Nrf2 is essential for protection against acute pulmonary injury in mice. Proc Natl Acad Sci USA 1999;96:12731–6.
39. Cho HY, Jedlicka AE, Reddy SP, et al. Role of NRF2 in protection against hyperoxic lung injury in mice. Am J Respir Cell Mol Biol 2002;26:175–82.
40. Aoki Y, Sato H, Nishimura N, Takahashi S, Itoh K, Yamamoto M. Accelerated DNA adduct formation in the lung of the Nrf2 knockout mouse exposed to diesel exhaust. Toxicol Appl Pharmacol 2001;173:154–60.
41. Oyasu R, Iwasaki T, Matsumoto M, Hirao Y, Tabuchi Y. Induction of tumors in heterotopic bladder by topical application of N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)nitrosamine. Cancer Res 1978;38:3019–25.
42. Zhang YY, Ludwikowski B, Hurst R, Frey P. Expansion and long-term culture of differentiated normal rat urothelial cells in vitro. In Vitro Cell Dev Biol Anim 2001;37:419–29.
43. Ozaki K, Sukata T, Yamamoto S, et al. High susceptibility of p53(+/−) knockout mice in N-butyl-N-(4-hydroxybutyl)nitrosamine urinary bladder carcinogenesis and lack of frequent mutation in residual allele. Cancer Res 1998;58:3806–11.
44. Kumagai Y, Lin LY, Hiratsuka A, et al. Participation of cytochrome P450–2B and -2D isozymes in the demethylenation of methylenedioxymethamphetamine enantiomers by rats. Mol Pharmacol 1994;45:359–65.
45. Bonfanti M, Magagnotti C, Bonati M, Fanelli R, Airoldi L. Pharmacokinetic profile and metabolism of N-nitrosobutyl-(4-hydroxybutyl)amine in rats. Cancer Res 1988; 48:3666–9.
46. Moon RC, Kelloff GJ, Detrisac CJ, Steele VE, Thomas CF, Sigman CC. Chemoprevention of OH-BBN-induced bladder cancer in mice by oltipraz, alone and in combination with 4-HPR and DFMO. Anticancer Res 1994;14:5–11.
47. Kang KW, Cho IJ, Lee CH, Kim SG. Essential role of phosphatidylinositol 3-kinase-dependent CCAAT/enhancer binding protein beta activation in the induction of glutathione S-transferase by oltipraz. J Natl Cancer Inst 2003;95:53–66.
48. Nelson CP, Kidd LC, Sauvageot J, et al. Protection against 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine cytotoxicity and DNA adduct formation in human prostate by glutathione S-transferase P1. Cancer Res 2001;61:103–9.
49. Nelson WG, De Marzo AM, Deweese TL, et al. Preneoplastic prostate lesions: an opportunity for prostate cancer prevention. Ann NY Acad Sci 2001;952:135–44.
50. Strassburg CP, Manns MP, Tukey RH. Differential down-regulation of the UDP-glucuronosyltransferase 1A locus is an early event in human liver and biliary cancer. Cancer Res 1997;57:2979–85.

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NUMERIC ABERRATIONS OF HER-2 AND CHROMOSOME 17 DETECTED BY FLUORESCENCE IN SITU HYBRIDIZATION IN URINE-EXFOLIATED CELLS FROM PATIENTS WITH UROTHELIAL CARCINOMA

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ABSTRACT

Objectives. To elucidate the clinical significance of the *HER-2* gene alterations in urine-exfoliated cells detected by fluorescence in situ hybridization (FISH) in patients with urothelial transitional cell carcinoma.

Methods. The relative increase of *HER-2* (RI-HER2) and gain of chromosome 17 (G-17) in urine-exfoliated cells were examined using DNA probes for *HER-2* and the chromosome 17 centromere in 103 patients. In addition, FISH analysis was performed using corresponding paraffin-embedded tissue sections from 45 cases to compare the results obtained using urine-exfoliated cells and those obtained using paraffin-embedded tissue.

Results. RI-HER2 and G-17 was found in 23 (22.3%) and 46 (44.6%) of 103 patients, respectively. RI-HER2 was significantly more frequent in tumors with two or more recurrences (40.7% versus 15.8%, $P = 0.010$) and in those with carcinoma in situ (CIS) (35.4% versus 15.9%, $P = 0.029$). G-17 was more frequent in high-grade tumors (69.1% versus 16.7%, $P = 0.032$), invasive tumors (63.6% versus 14.3%, $P < 0.001$), and in patients with CIS (77.1% versus 29.0%, $P < 0.001$). The positive rate for FISH (presence of RI-HER2 and/or presence of G-17) tended to be more frequent in FISH than in cytology. A comparison of the analyses using urine-exfoliated cells and paraffin-embedded tissue showed identical results in 36 (80.0%) of 45 cases.

Conclusions. Numeric alterations of the chromosome 17 centromere in urine-exfoliated cells detected by FISH may reflect the malignant potential of urothelial carcinoma. In addition, a relative increase in *HER-2* was associated with the number of recurrences and the presence of CIS. UROLOGY **64:** 617–621, 2004. © 2004 Elsevier Inc.

Urinary cytology is primarily used in routine clinical practice to diagnose and monitor urothelial carcinoma. Although urinary cytology is a convenient and nontraumatic maneuver, its sensitivity largely depends on the ability of individual cytoscreeners. To supplement the performance of urinary cytology, a variety of diagnostic measures such as bladder tumor antigen (BTA)-stat and urinary nuclear matrix protein-22 (NMP-22) have been developed.[1] Multicolor fluorescence in situ hybridization (FISH) using chromosome 3, 7, 9p, and 17 DNA probes has been reported to be more sensitive and specific to urine-exfoliated cells than urinary cytology.[2] However, these diagnostic measures cannot be used to evaluate accurately the biologic behavior of carcinoma cells because the targets are not genes.

The proto-oncogene *HER-2*, located on chromosome 17q21, encodes tyrosine kinase growth factor receptor and regulates cell growth and differentiation.[3] It has been reported that the amplification of *HER-2* or overexpression of its product is associated with malignant cell transformation and a poor prognosis in a variety of tumors, such as gastric,[4] ovarian, and breast carcinomas.[5] In urothelial carcinomas, the amplification of *HER-2* has been detected by tissue immunohistochemistry,[6,7] Southern blotting,[7,8] polymerase chain reaction,[9,10] and FISH.[11–13] It has also been associated with tumor

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