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.

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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

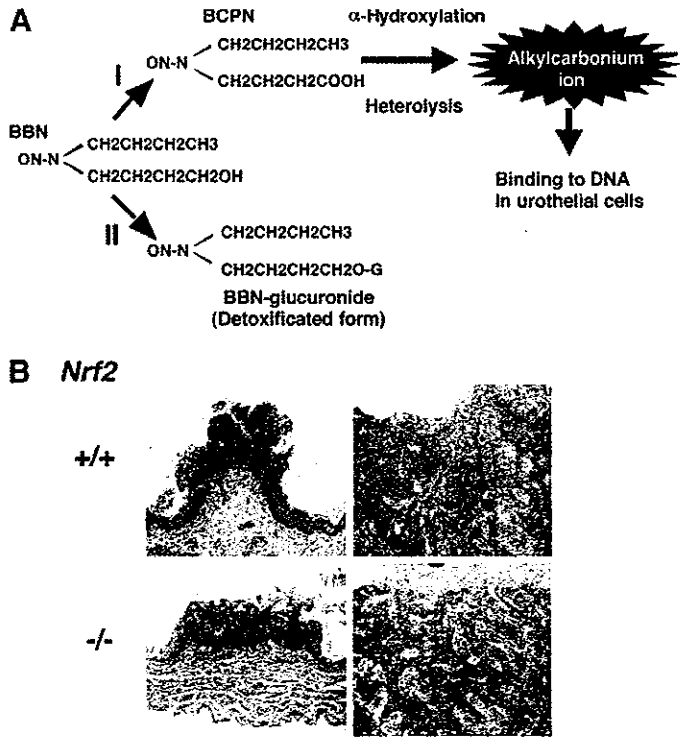

Fig. 1. BBN-induced carcinogenesis in wild-type and *Nrf2*$^{-/-}$ mouse urinary bladders. *A*, biotransformation processes of BBN; *G*, glucuronic acid. *B*, histopathologic analysis of tumor regions. Tissue sections of urinary bladder from wild-type (*top*) and *Nrf2*$^{-/-}$ (*bottom*) mice were analyzed 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

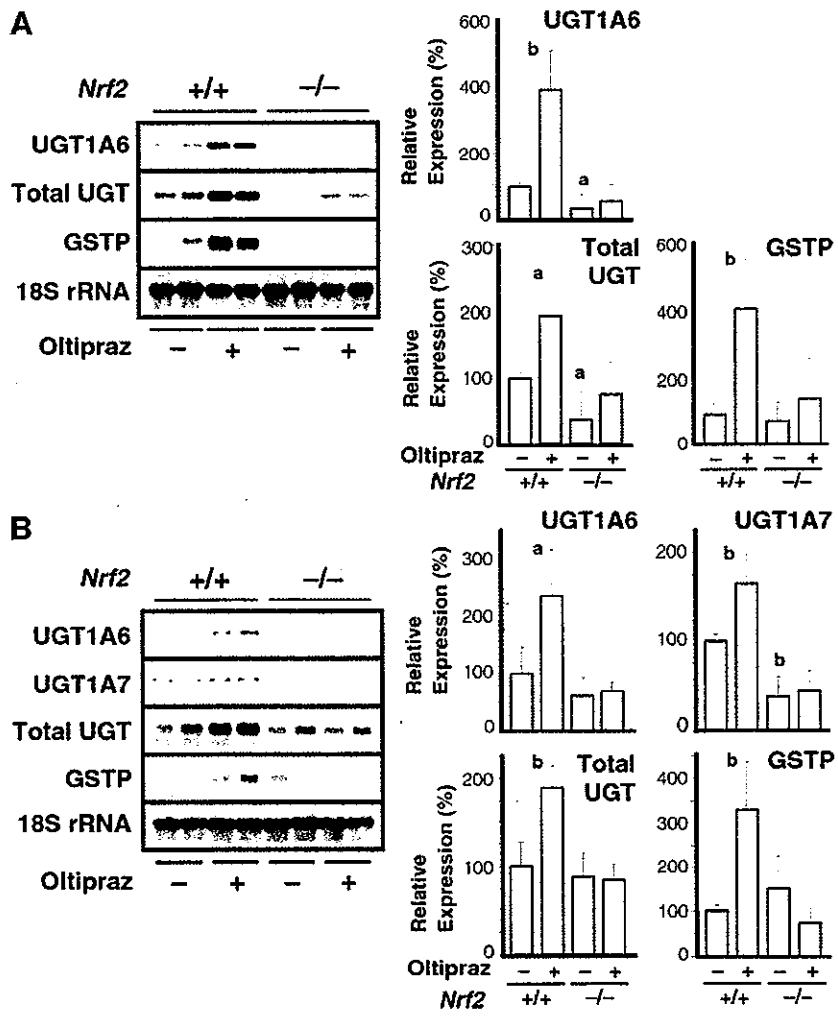

Fig. 2. Effect of oltipraz on the expression of phase 2 enzyme mRNAs in the liver and urinary bladder. *A* and *B*, effect of oltipraz on the expression of phase 2 enzyme genes in the liver (*A*) and urinary bladder (*B*) in wild-type and *Nrf2*$^{-/-}$ male mice. Oltipraz was fed at the concentration 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

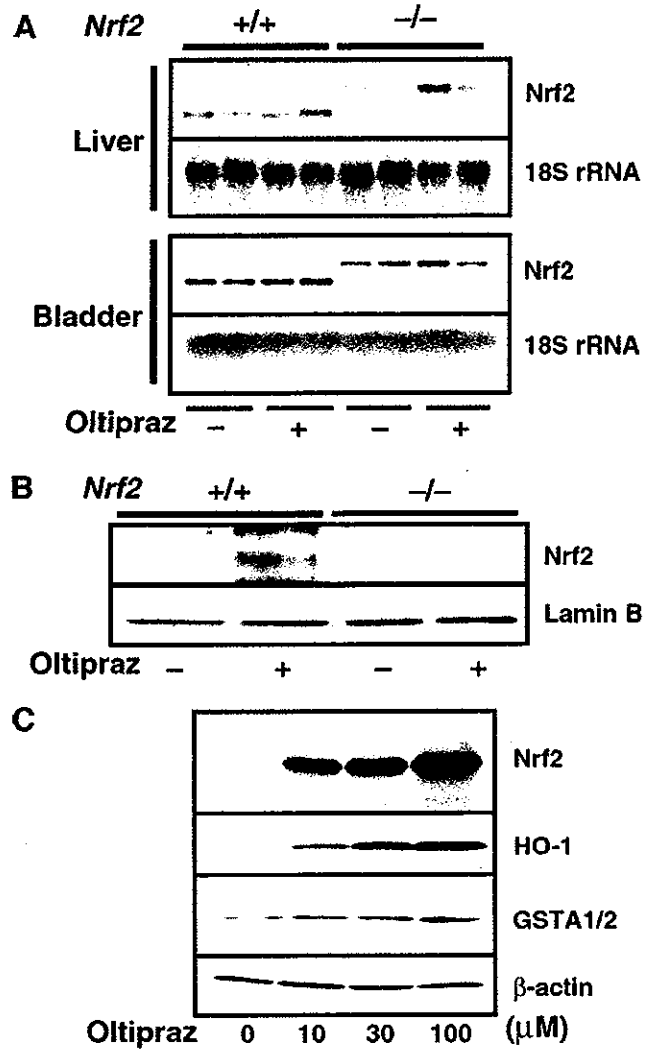

Fig. 3. Effect of oltipraz on Nrf2 activation in the liver and urinary bladder. *A*, effect of oltipraz on the expression of Nrf2 mRNA in the liver and urinary bladder of male wild-type and *Nrf2*$^{-/-}$ mice. Mice were fed oltipraz at the concentration of 1 g/kg diet for 48 hours. Densitometric analysis 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.

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*).

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-

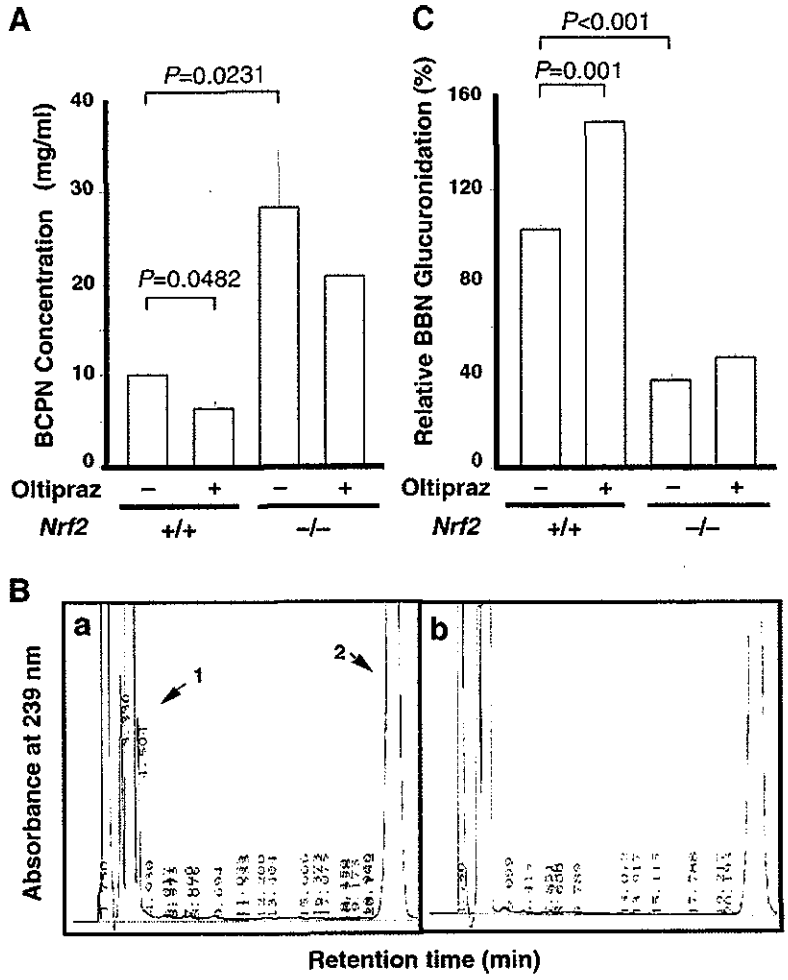

Fig. 4. Effect of *Nrf2* genotype and oltipraz treatment on the urinary concentration of BCPN after treatment with BBN and the activity of BBN glucuronidation in hepatic microsomes. *A*, Mice were fed 250 mg/kg diet of oltipraz and 0.05% BBN in drinking water for 2 weeks, and then urine samples were 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

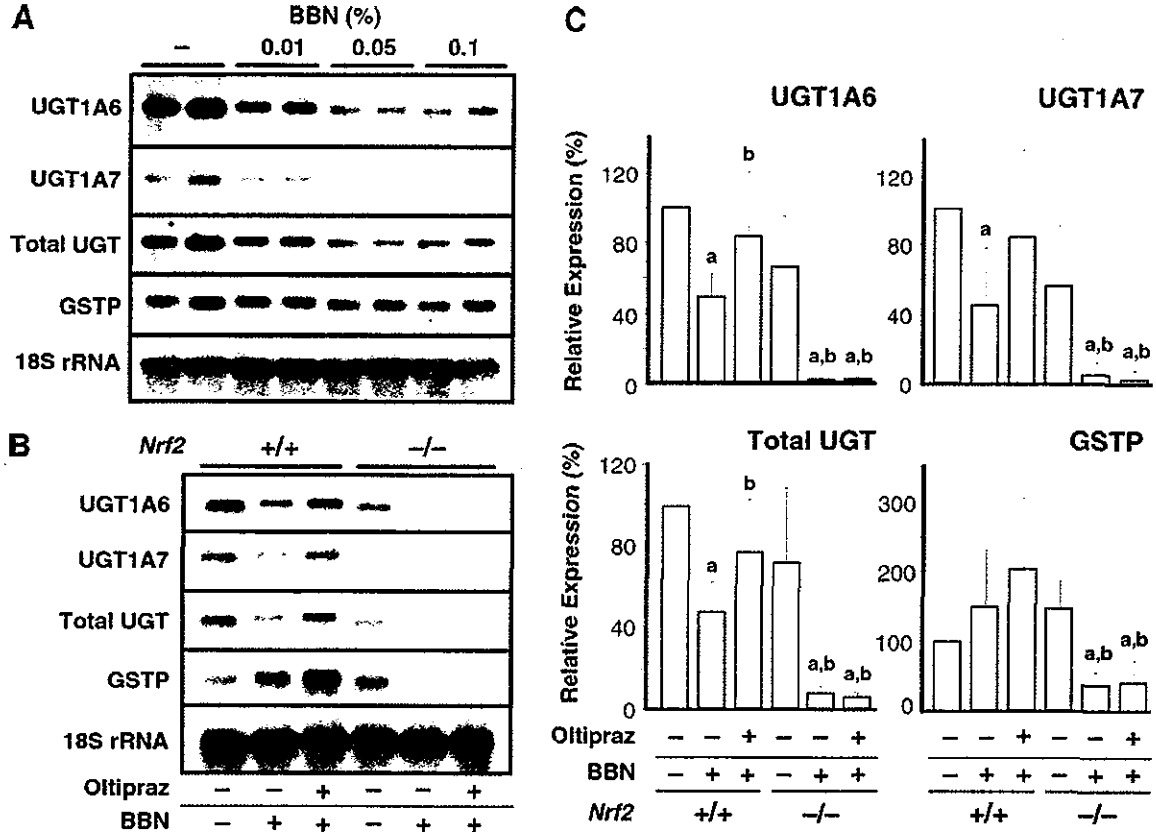

Fig. 5. Effect of BBN and oltipraz on the expressions of phase 2 genes. *A*, effect of BBN on the expressions of phase 2 genes in the urinary bladder of male wild-type mice. Mice were treated with 0.01%, 0.05%, 0.1% BBN, or vehicle in drinking water for 2 weeks. *B*, The expressions of phase 2 genes 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

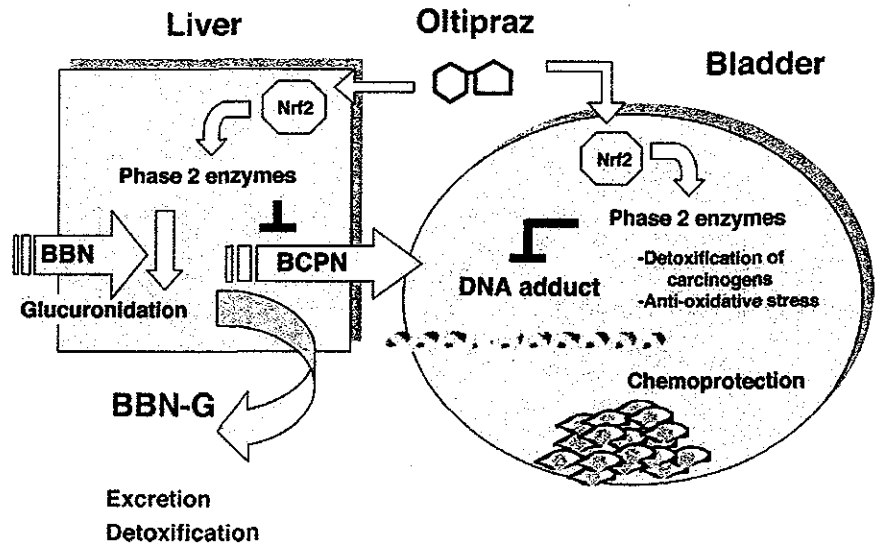

Fig. 6. Mechanism of chemoprotection by oltipraz against urinary bladder carcinogenesis. Oltipraz reduced BBN-induced carcinogenesis by suppressing the urinary excretion of BCPN by means of Nrf2-dependent induction of BBN glucuronidation in the liver. Moreover, oltipraz also works in the urinary bladder 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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ELSEVIER

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

TAKAMITSU INOUE, KAZUNARI SATO, NORIHIKO TSUCHIYA, SHINOBU MATSUURA, MASAHIRO IINUMA, TOMONORI HABUCHI, AND TETSURO KATO

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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TABLE I. *Demographic data for 103 patients*

Characteristic	
Average age (yr)	72.20 ± 10.62
Sex	
Male	74 (71.8)
Female	29 (28.2)
Carcinoma site	
Upper urinary tract	12 (11.7)
Bladder	91 (88.3)
Operation	
TURBT	63 (61.2)
Total cystectomy	19 (18.4)
Nephroureterectomy ± cystectomy	14 (13.6)
Partial cystectomy or ureterectomy	2 (1.9)
Biopsy alone (no surgery)	5 (4.8)
Tumor stage	
pTis	15 (14.8)
pTa	42 (41.6)
pT1	17 (16.9)
pT2–T4	27 (26.7)
Tumor grade	
1	14 (13.6)
2	34 (33.0)
3	55 (53.4)
Carcinoma in situ	
Positive	31 (30.1)
Negative	72 (69.9)
Tumor growth pattern	
Papillary	65 (63.1)
Nodular	23 (22.3)
Flat (pTis)	15 (14.6)
Recurrence	
Initial presentation (0)	60 (58.3)
First recurrence (1)	16 (15.5)
Two or more recurrences (≥2)	27 (26.2)

KEY: TURBT = transurethral resection of bladder tumor.
Data presented as number of patients, with the percentage in parentheses, unless otherwise indicated.

grade, tumor stage, and the patient's outcome.[7,9,10] These studies, however, evaluated isolated nuclei from frozen tissues[8,11] or paraffin-embedded tissue sections.[7,9,10,12] Urine-exfoliated cells would be usable in FISH, which might be able to evaluate *HER-2* alterations directly and independently.

To determine the clinical significance and applicability of *HER-2* alterations in urine-exfoliated cells, we examined the numeric aberrations of *HER-2* in samples from patients with urothelial carcinomas using FISH.

MATERIAL AND METHODS

PATIENTS

A total of 103 patients, who were pathologically diagnosed with urothelial transitional cell carcinoma at the Akita University Medical Center and Hiraka General Hospital from 1999 to 2002, were entered in this study. The demographic data for the 103 patients are shown in Table I. Of the 103 patients, 91 had bladder carcinoma and 12 renal pelvic or ureteral carcinoma; 60 had tumors of initial presentation and 43 recurrent tumor; and 68 had papillary tumor and 23 nodular tumor. Of the 31 patients with pathologically confirmed carcinoma in situ (CIS; pTis), 15 did not have exophytic tumors (primary CIS; pTis) and 6 had tumor-associated CIS (concomitant CIS). Of the 103 patients, 98 underwent surgical intervention and 5 underwent random biopsy of the bladder alone. The tumor grade was available for all 103 patients, and the pathologic stage was available for 101 patients. The remaining 2 cases were excluded from pathologic staging because of inadequate specimens. The tumor grade and pathologic stage were determined by the general guidelines issued by the Japanese Urological Association, which are based mainly on the World Health Organization criteria.[14,15] In this study, grade 1-2 was considered low grade and grade 3 high grade, and pTa was described as superficial and pT1-T4 as invasive. The Internal Ethical Board of Akita University approved this study, and all patients provided written informed consent.

URINE PREPARATION FOR FISH

About 20 to 100 mL of a spontaneously voided urine sample was obtained before surgery and separated into two tubes, one for FISH and one for urinary cytology. Within 3 hours after sampling, the urine cells were centrifuged at 3000 rpm for 10 minutes. The cell pellet was rinsed with phosphate-buffered saline, fixed with a methanol/acetic acid 3:1 solution (Carnoy's solution), and re-suspended in about 300 μL of Carnoy's solution. An aliquot of the cell suspension was placed on a 12-well slide (Shandon, Pittsburgh, Pa) and dried overnight.

DNA PROBES FOR FISH

FISH was performed using dual-color DNA probes that hybridize to the band region of 17q11.2-q12 (LSI *HER-2/neu* SpectrumOrange, Vysis, Downers Grove, Ill) and 17p11.1-q11.1, locus D17Z1 (Chromosome Enumeration Probe 17 [CEP17], SpectrumGreen, Vysis).

FISH OF URINE-EXFOLIATED CELLS

The cells in the 12-well slide were incubated in 2 × saline/sodium citrate (SSC) at 37°C for 10 minutes, 0.5 mg/mL pepsin at 37°C for 13 minutes, phosphate-buffered saline for 10 minutes, 1% formaldehyde for 5 minutes, and phosphate-buffered saline for 10 minutes. They were then dehydrated in ethanol, air-dried, denatured in 2 × SSC/70% formamide at 73°C for 5 minutes, and again dehydrated in ethanol. The cells were incubated with 3 μL of the denatured FISH probe mixture (probe mixture to hybridization buffer to water 1:7:2) at 37°C overnight in a humidified chamber and then rinsed in 0.4% SSC/0.3% NP-40 at 73°C for 2 minutes and 2 × SSC/0.1% NP-40 at room temperature for 1 minute. The cell nuclei were counterstained with 3 μL of 4,6-diamino-2-phenylindole.

URINARY CYTOLOGY

Urinary specimens were stained using the Papanicolaou method and evaluated by cytoscreeners at the Akita University Medical Center according to the five-grade classification. Class V was defined as positive cytology findings.

FISH OF PARAFFIN-EMBEDDED TISSUE SECTIONS

FISH of the tissue sections was performed as described previously.[16] In brief, 5-μm-thick tissue sections from each paraffin-embedded tissue block were put onto slides, deparaffinized, and dehydrated. The slides were microwaved in 10 mM citric acid (pH 6.0) for 10 minutes and incubated in 4 mg/mL of pepsin at 37°C for 13 minutes. Hybridization was done at 80°C for 2 minutes, 50°C for 30 minutes, and 37°C overnight in a humidified chamber with 3 μL of the denatured

TABLE II. *Number of copies for* **HER-2** *and chromosome 17 centromere among 11 urine samples from patients with nonmalignant disease (control)*

Probe	Copy No.				
	0	1	2	3	≥4
HER-2	0.00 ± 0.00 (0–0)	0.55 ± 3.87 (0–4)	199.36 ± 3.84 (196–200)	0.09 ± 0.91 (0–1)	0.00 ± 0.00 (0–0)
CEP17	0.00 ± 0.00 (0–0)	0.64 ± 4.50 (0–5)	199.18 ± 4.62 (195–200)	0.18 ± 1.22 (0–1)	0.00 ± 0.00 (0–0)

KEY: CEP17 = Chromosome Enumeration Probe 17; No. = number.
Data presented as mean ± 3 standard deviations, with the range in parentheses.

FISH probe mixture. The slides were washed in 1.5 M urea/0.1 × SCC at 45°C for 10 minutes three times. Finally, the cell nuclei were stained with 3 μL of 4,6-diamino-2-phenylindole.

SCORING OF FISH SIGNALS

FISH signals were evaluated under an Olympus BX10 microscope (Olympus, Tokyo, Japan) equipped with filters for Texas Red, FITC, and 4,6-diamino-2-phenylindole and a digital camera (DXM1200, Nikon, Tokyo, Japan). The normal value study was performed by enumerating the *HER-2* and CEP17 signals in urine-exfoliated cells from 11 patients who proved not to have any pathologic findings in the urinary tract. Of these age-matched 11 patients, 6 were men and 5 were women; their disease condition was benign prostatic hyperplasia in 4, chronic renal failure in 4, urolithiasis in 2, and erectile dysfunction in 1. On the basis of the normal value study (Table II), the numeric aberration of the FISH signals in the urine-exfoliated cells was categorized as a gain of chromosome 17 (G-17) and a relative increase in *HER-2* (RI-HER2). The RI-HER2 category, which contains overrepresentation (eg, duplication, triplication) and amplification of *HER-2*, was defined as having at least four nuclei with a *HER-2*/CEP17 ratio of 2.0 or more and two or more signals for CEP17. The G-17 category was defined as having at least four nuclei with three or more signals for CEP17. The samples with RI-HER2 and/or G-17 were described as FISH positive. Up to 100 nuclei of cytologically atypical features, such as nuclear enlargement or irregular nuclear contour for the urine specimens and pathologic carcinoma cells for the tissue sections, were scored in each case. When at least four nuclei were classified as RI-HER2 or G-17 positive, no more cells were counted. However, in cases that were FISH negative, we routinely counted at least 100 cells to exclude false-negative results.

In the evaluation of FISH of the tissue sections, more than 10% of the nuclei with three or more same-color signals were required for the categorical classification of a numeric aberration and thus to be considered FISH positive.

STATISTICAL ANALYSIS

Fisher's exact test or the chi-square test was used to examine the relationship between RI-HER2, G-17, FISH-positive findings, and the cytologic parameters. A P value less than 0.05 was considered statistically significant.

RESULTS

The FISH and cytologic examinations of the exfoliated cells were performed for all 103 patients. The average number of counted cells per FISH specimen was 50.0 (range 8 to 100). The average *HER-2*/CEP17 ratio in the RI-HER2 and G-17 categories was 2.13 and 1.13, with an average *HER-2* signal number per nucleus of 6.04 and 4.39, respectively. The frequencies of RI-HER2 and G-17 are listed in Table III.

RI-HER2 and G-17 was found in 23 (22.3%) and 46 (44.6%) of 103 urine specimens, respectively. RI-HER2 tended to be more frequently found in recurrent tumors than in tumors of initial presentation (30.2% versus 16.6%, $P = 0.149$). RI-HER2 was significantly more frequent in the tumors with two or more recurrences compared with those of initial presentation or a first recurrence (40.7% versus 15.8%, $P = 0.010$) and in those with CIS than in those without CIS (35.4% versus 15.9%, $P = 0.029$; Table III). However, RI-HER2 was not associated with tumor grade, stage, or growth pattern. G-17 was significantly more frequent in high-grade than low-grade (69.1% versus 16.7%, $P = 0.032$), invasive than superficial (63.6% versus 14.3%, $P < 0.001$), CIS-positive than CIS-negative (77.1% versus 29.0%, $P < 0.001$), and nodular than papillary (65.2% versus 30.8%) tumors ($P = 0.004$; Table III). However, G-17 was not associated with the number of recurrences.

When "FISH positive" was defined as the presence of RI-HER2 and/or G-17, FISH was positive in 59 (57.3%) of the 103 cases. The positive rate was significantly greater in the high-grade tumors (78.2% for G3 versus 31.2% for G1-G2, $P < 0.001$), invasive tumors (70.5% for pT1-T4 versus 33.3% for pTa, $P = 0.001$), and in the presence of CIS (90.3% for tumors with CIS versus 42.0% for those without CIS, $P = 0.010$; Table III). When compared with urinary cytology, the positive results tended to be more frequently found using FISH than using cytology (57.3% versus 45.6%, $P = 0.094$; Table III).

FISH of the corresponding tissue sections was successfully performed in 45 cases. When the positive or negative FISH category was applied and compared between the results from the urine-exfoliated cells and those from the tissue sections, 36 (80.0%) of the 45 cases had the same result.

COMMENT

We selected *HER-2* as a genetic marker of urothelial carcinoma cells because this gene is potentially

TABLE III. *Cytology and FISH results of urine-exfoliated cells and tumor characteristics*

Tumor Characteristic	n	RI-HER2 (%)	G-17 (%)	RI-HER2 and/or G-17 (%) (FISH Positive)	Cytology (%)	*P* Value (FISH Positive vs. Cytology)
Total	103	23 (22.3)	46 (44.6)	59 (57.3)	47 (45.6)	0.094
Grade						
1	14	3 (21.4)	1 (7.1)	4 (28.6)	0 (0.0)	0.256
2	34	6 (17.6)	7 (20.6)	12 (35.3)	11 (32.3)	
3	55	14 (25.5)	38 (69.1)	43 (78.2)	36 (65.5)	0.138
P value (G1–G2 vs. G3)		0.482	0.032	<0.001	<0.001	
Stage*						
pTa	42	8 (19.0)	6 (14.3)	14 (33.3)	6 (14.3)	0.173
pT1–T4	44	9 (20.5)	28 (63.6)	31 (70.5)	28 (63.6)	0.496
P value (pTa vs. pT1–T4)		0.999	<0.001	0.001	<0.001	
Carcinoma in situ*						
Positive	31	11 (35.4)	24 (77.1)	28 (90.3)	24 (77.4)	0.167
Negative	69	11 (15.9)	20 (29.0)	29 (42.0)	22 (31.9)	0.217
P value (positive vs. negative)		0.029	<0.001	0.010	<0.001	
Growth pattern						
Papillary	65	14 (21.5)	20 (30.8)	31 (47.7)	21 (32.3)	0.073
Nodular	23	4 (17.3)	15 (65.2)	16 (69.6)	14 (60.9)	0.400
P value (papillary vs. nodular)		0.668	0.004	0.071	0.016	
Recurrence						
Initial presentation (0)	60	10 (16.6)	31 (51.7)	36 (60.0)	33 (55.0)	0.416
First recurrence (1)	16	2 (12.5)	6 (37.5)	7 (43.8)	5 (31.2)	
Two or more recurrences (≥2)	27	11 (40.7)	9 (33.3)	16 (59.3)	9 (33.3)	0.056
P value (0–1 vs. ≥2)		0.010	0.168	0.810	0.135	

Key: RI-HER2 = relative increase of HER-2 *gene; G-17 = gain of chromosome 17; FISH = fluorescence in situ hybridization.*
** Statistical analysis performed for 101 patients.*

implicated in urothelial carcinogenesis.[6–10] Using FISH, previous studies have demonstrated *HER-2* "amplification" in 3.4% to 7.0% of urothelial carcinomas and a "gain or relative increase" in 8.5% to 41.4%.[11–13] However, none of these studies evaluated the copy number of *HER-2* and chromosome 17 on urine-exfoliated cells using FISH. In the present study, an RI-HER2 was found in 22.3% of the cases. Although the FISH criteria were considerably different among the individual studies, the frequency of the RI-HER2 for the urine-exfoliated cells as shown by our study was comparable to that for the tissue specimens reported by the others.[11–13]

In 36 (80%) of 45 cases, the FISH findings were the same in the urine-exfoliated cells as in the tissue sections. This may indicate the validity of using urine-exfoliated cells, as well as the good reproducibility of the FISH analysis. Of the 9 cases in which the results were not the same, 8 were low-stage (pTa-T1) tumors, and 7 of the 9 cases were positive using the urine samples and negative using paraffin-embedded tissue, suggesting a greater sensitivity for the urine-exfoliated samples. The discrepancy might have stemmed from a low tumor burden, poor exfoliation of tumor cells, genetic heterogeneity among different cell clusters, or combinations thereof.[17] The present findings suggested that the abnormal genetic characteristics of urothelial tumors can be determined in most cases by examining urine-exfoliated cells.

Of the alterations detected with the FISH analysis using exfoliated cells, RI-HER2 tended to be more frequently found in recurrent tumors than in tumors of initial presentation and was significantly more frequent in the tumors with two or more recurrences. Interestingly, although RI-HER2 was infrequently found in patients with grade 1, all three grade 1 tumors with RI-HER2 were those with two or more recurrences. An RI-HER2 in urine specimens, therefore, may reflect a distinct potential for recurrence even in low-grade tumors.

In this study, both RI-HER2 and G-17 were more frequently observed in cases with CIS than in those without. However, the presence of RI-HER2 did not correlate with tumor grade or stage, and G-17 correlated significantly. In partial support of our findings, Ohta *et al.*[11] reported that no correlation was found between the relative increase in the *HER-2* copy number and tumor stage but that the gain in chromosome 17 correlated more significantly with tumor grade and stage. Sauter *et al.*[12] also showed that *HER-2* amplification, found more frequently in pT2-T4 tumors than in pTa-T1, did not correlate with tumor grade and was found only in tumors with aneusomy of chromosome 17. In

our study, G-17 was more strongly associated with the presence of CIS than was RI-HER2 (Table III). When grade 3 tumors with CIS were compared with grade 3 tumors without CIS, no difference in the RI-HER2 frequency was found ($P = 0.999$). Also, when tumors without CIS and those with primary CIS (pTis) were compared, no statistically significant difference was found in the RI-HER2 frequency ($P = 0.160$). Therefore, our statistically significant association between RI-HER2 and the presence of CIS might have derived from the strong association between G-17 and CIS. Furthermore, the results of our study and others[11,12] may indicate that other genes on chromosome 17 play an additional significant role in progression, although *HER-2* may be partly involved in the progression of urothelial cancer.

A recent study using Urovision, which includes chromosome 17 centromere probes, showed that numeric alterations of chromosome 17 correlated significantly with tumor recurrence.[18] In the present study, although the high positive rate of FISH was obviously derived from chromosome 17 centromere analysis alone, the addition of the relative increase in the number of *HER-2* copies increased the positive rate from 77% to 90% in the presence of CIS (Table III). Furthermore, although G-17 was not associated with the rate of recurrence, the RI-HER2 may be significantly associated with multiple recurrences (Table III). This suggests that the *HER-2* increase associated with a chromosome 17 gain may reflect an accumulation of genetic alterations involving the development of CIS, which has a high probability of progressing to invasive carcinoma.[19] Furthermore, RI-HER2, which presumably leads to overexpression of HER-2,[12] may be biologically related to recurrence in the heterotopic urothelium independent of a gain of chromosome 17.

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, the relative increase of the *HER-2* copy number may be associated with the number of recurrences and the presence of CIS. Periodic FISH analysis of urine samples could be used to determine whether the *HER-2* alterations occurred sequentially or were enhanced in individual cases.

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Original Article

Impact of adjuvant systemic chemotherapy on postoperative survival in patients with high-risk urothelial cancer

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Abstract

Background: The objective of this study was to retrospectively investigate the effectiveness of adjuvant combination chemotherapy for locally advanced urothelial cancer.

Methods: Between 1987 and 1998, 56 patients with locally advanced bladder ($n = 27$) or upper urinary tract ($n = 29$) cancer (pathological stage T3, T4 or N1, N2 and M0) were treated by radical cystectomy or radical nephroureterectomy and regional lymphadenectomy. Thirty-one patients had lymph node-positive disease and 25 patients did not. Twenty patients underwent adjuvant chemotherapy and 36 patients were observed after surgery. Cox proportional hazards models were used to determine the impact of numerous clinicopathological findings on survival. A subgroup analysis of patients with lymph node-positive disease was conducted to evaluate disease-free survival and overall survival rates.

Results: In this series, the median follow-up period was 39 months (range, 4–163) after surgery. Disease-free and overall survival rates of all 56 patients were 45% and 58%, respectively, at 3 years. Only lymph node status was significantly associated with disease-free and overall survival in the multivariate analyses. In a subgroup analysis of patients with lymph node-positive disease, 16 patients who underwent adjuvant chemotherapy had superior disease-free survival compared to 15 patients with no adjuvant chemotherapy ($P = 0.0376$).

Conclusion: These findings show that the prognosis of advanced urothelial cancer is significantly associated with nodal status. Furthermore, adjuvant combination chemotherapy has a positive impact on survival in patients with lymph node-positive disease.

Key words adjuvant chemotherapy, pathological positive node, urothelial cancer.

Introduction

The prognosis of patients with locally advanced urothelial cancer is poor. The aggressive nature of this cancer, and the associated high morbidity and mortality, warrant appropriate therapeutic intervention. Although the optimal treatment for the individual patient with advanced urothelial cancer is still unclear, radical surgery (e.g. radical cystectomy for locally advanced bladder cancer or radical nephroureterectomy for advanced upper urinary tract cancer) is considered to be the standard treatment option for selected patients with a long life expectancy. In fact, the 5-year overall survival rate in patients who undergo radical surgery is approximately 50–60%. However, clinical outcomes of these patients are not uniform. To improve the prognosis of these urothelial cancer patients, some additional treatment along with radical surgery is needed. Since the report by Sternberg *et al.*, systemic chemotherapy consisting of methotrexate, vinblastine, adriamycin and cisplatin (M-VAC), has been shown to be effective for advanced urothelial cancer.[1] Several reports have described the

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positive clinical impact of adjuvant systemic chemotherapy after radical surgery.[2-4] However, it is still unknown whether adjuvant systemic chemotherapy improves the prognosis of locally advanced urothelial cancer. Clearly, if it does, we have to make clear feasible selection criteria to determine which patients should undergo adjuvant chemotherapy.

This retrospective analysis of our experience with adjuvant systemic chemotherapy was conducted using the medical records of patients who underwent radical surgery for locally advanced and/or node-positive urothelial cancer of the bladder and upper urinary tract. Our aims were to gain further insight into the positive survival impact of adjuvant systemic chemotherapy on this disease and to guide selection of patients who might benefit most from this multimodal approach.

Patients and methods

Of the patients with advanced urothelial cancer who underwent either radical cystectomy and bilateral pelvic lymphadenectomy or radical nephroureterectomy and lymphoadenectomy at our institute from 1987 to 1998, 56 fulfilled the eligibility criteria for the present study. The eligibility criteria included: (i) pathological stage T3 or more, based on the 1997 tumor, nodes, and metastasis (TNM) system for histopathological tumor staging of bladder and upper urinary tract cancer; (ii) pathologically defined nodal disease regardless of the pathological stage of the primary tumor; (iii) lack of evidence of macroscopic residual disease and distant metastases; and (iv) good performance status (PS 0 or 1). The routine lymphadenectomy included the bilateral obturator and iliac lymph nodes in patients with cancer of the bladder or lower part of the ureter, and the unilateral retroperitoneal lymph nodes in patients with cancer of the renal pelvic or upper and middle part of the ureter. We excluded patients with pathological stage T2 or less who had both bladder cancer and upper urinary tract cancer without nodal metastasis, patients with distant metastasis, patients who received neoadjuvant chemotherapy or preoperative radiation therapy, and patients with renal-, hepatic- or myelo-dysfunction.

Although the indications were not defined precisely, the decision to administer adjuvant chemotherapy to these high-risk patients was based on patient acceptance after informed consent. Adjuvant chemotherapy consisted of methotrexate, vinblastine, adriamycin and cisplatin (M-VAC) or methotrexate, epirubicin and cisplatin (MEC).[1,5] Follow-up examinations were performed four times in the first 2 years, semiannually for the next 3 years, and annually thereafter, or as clinically indicated. Follow-up examinations of both bladder cancer patients and upper urinary tract cancer patients included physical examination with laboratory tests, chest X-ray, computed tomography (CT) of the abdomen and the pelvis, and cytological examination of urine. Bone scintigraphy and chest CT were performed if indicated clinically. For the patients with upper urinary tract cancer, cystoscopy was performed in addition to the other follow-up examinations at these visits. Although the decision for additional treatment was open when a tumor recurred, most affected patients received several courses of systemic chemotherapy (M-VAC or MEC).

Disease-free survival and overall survival times were recorded from the date of radical surgery to the date of documented recurrence or death, as were all causes of death. Patients who had not relapsed, or were alive with/without cancer were censored. Cox proportional hazards models were used to determine the prognostic significance of numerous clinical and pathological findings using disease-free survival and overall survival as the end points. Significant tests were based on the test score for a Cox proportional model. Stepwise variable selection was used, with a P-value of 0.05 or less required to enter the model. Survival curves were obtained using the Kaplan–Meier method and were compared with the use of the log-rank test. A P-value less than 0.05 was considered statistically significant and all P-values were two-sided.

Results

Patient and tumor characteristics are detailed in Table 1. The mean age at operation was 65.1 years (median, 66 years; range, 41–85 years). Forty-six of the patients were men and 10 were women. The primary cancer site was the urinary bladder in 27 patients and the upper urinary tract in 29 (renal pelvis in eight, ureter in 16, and both in five patients). Performance status was 0 or 1 in these patients. The mean follow-up period was 49.4 months (median, 39.5 months; range, 4–163 months). Histological examination of these patients demonstrated urothelial (transitional cell) carcinoma in 50 patients, urothelial carcinoma plus adenocarcinoma in three, urothelial carcinoma plus squamous cell carcinoma in one, and urothelial carcinoma plus adenocarcinoma plus squamous cell carcinoma in two. The pathological grade of operated specimens was Grade 2 in 17 patients and Grade 3 in 39 patients. Although the pathological stage (pT) of bladder cancer was T1 in one patient, T2 in two, T3a in one, T3b in 16 and T4 in seven patients, that of upper urinary tract cancer was T1 in

one, T2 in one, T3 in 22 and T4 in five patients. Thirty-one patients had node-positive disease (N1 in 12 and N2 or more in 19) and 25 patients had node-negative disease. Twenty patients underwent adjuvant chemotherapy after surgery and the remaining 36 patients did not. M-VAC was given to 17 patients (1–3 cycles, median two cycles) and MEC was given to three patients (three cycles in each patient). The median interval from surgery to adjuvant chemotherapy was 1 month (range, 1–3 months).

Disease-free and overall survival rates of all 56 patients were, respectively, 45% and 58% at 3 years, and 41% and 46% at 5 years.

Table 1 Baseline patient and tumor feature

	n
Gender	
Male	46
Female	10
Age (years)	
Range	41–85
Median	66
Primary site	
Upper urinary tract	29
Bladder	27
Tumor histology	
Pure urothelial cancer	50
Others	6
Tumor grade	
G2	17
G3	39
Pathological stage	
pT1†	2
pT2†	3
pT3	39
pT4	12
Nodal status	
N0	25
N1	12
N2	19

†These cases had nodal disease.

Gender, age, primary site, tumor histology, pathological grade, lymph node status, and adjuvant chemotherapy were included in the multivariate analysis. The most significant risk factor that predicted both disease-free and overall survival was lymph node status. Adjuvant chemotherapy reached a marginally significant level for disease-free survival ($P = 0.051$) (Table 2).

In 31 patients with lymph node-positive disease, which was the most significant risk factor, we analyzed whether adjuvant chemotherapy had a positive survival benefit or not. Baseline characteristics of the 31 patients are presented in Table 3. There was a similar distribution of gender, age, P and N stage and tumor histology in both groups. Disease-free and overall survival rates in 16 patients who underwent adjuvant chemotherapy were 56% and 63% at 3 years, respectively, whereas disease-free and overall survival rates for 15 patients with no adjuvant chemotherapy were 10% and 27%, respectively (Fig. 1). A significant difference in disease-free survival was shown between patients with adjuvant chemotherapy and those without it ($P = 0.0376$). There was a slight, non-significant, difference in overall survival between the two groups.

Discussion

In the present study, we showed that the prognosis of advanced urothelial cancer was significantly associated with nodal status in patients with these tumors. Adjuvant chemotherapy did not affect the survival of all patients together. However, in subgroup analysis of patients with node-positive disease, adjuvant chemotherapy had a positive survival benefit.

Locally advanced bladder cancer is defined as muscle-invasive tumor growth beyond the bladder wall and/or involving regional lymph nodes, which includes pelvic lymph nodes below the aortic bifurcation (tumor stages pT3a, pT3b, pT4 and/or pN+ and M0, 1997 TNM classification). Locally advanced upper urinary tract

Table 2 Multivariate analysis of factors associated with survival in 56 patients

Factors	Variables	Disease-free survival		Overall survival	
		P-value	Risk ratio (95% CI)	*P*-value	Risk ratio (95% CI)
Gender	Male/female	0.068	0.437 (0.180–1.063)	0.335	0.626 (0.242–1.620)
Age	≥65/<65	0.192	1.685 (0.770–3.691)	0.059	2.281 (0.968–5.368)
Primary site	UUT/B	0.239	0.625 (0.286–1.366)	0.170	0.577 (0.263–1.266)
Tumor histology	Others/pure UC	0.104	0.288 (0.064–1.291)	0.206	0.381 (0.085–1.700)
Tumor grade	G3/G2	0.528	1.295 (0.581–2.885)	0.992	1.044 (0.435–2.510)
Nodal disease	N+/N0	0.015	2.843 (1.226–6.592)	0.012	3.077 (1.281–7.390)
Adjuvant chemotherapy	No/Yes	0.051	2.367 (0.996–5.631)	0.257	1.660 (0.691–3.987)

B, bladder; CI, confidence interval; UC, urothelial cancer; UUT, upper urinary tract.

Table 3 Baseline characteristics of patients with lymph-node positive disease

	Adjuvant chemotherapy ($n = 16$)	Surgery alone ($n = 15$)	P-value
Gender			
Male	14	13	
Female	2	2	>0.9999
Age (years)			
Range	41–78	47–84	
Median	56	67	0.1198
Primary site			
UUT	8	5	
B	8	10	0.4725
Tumor histology			
Pure UC	14	13	
Others	2	2	>0.9999
Tumor grade			
G2	2	4	
G3	14	11	0.3944
Pathological stage			
pT1	0	2	
pT2	2	1	
pT3	11	8	
pT4	3	4	0.307
Nodal status			
N1	6	6	
N2	10	9	>0.9999

B, bladder; UC, urothelial cancer; UUT, upper urinary tract.

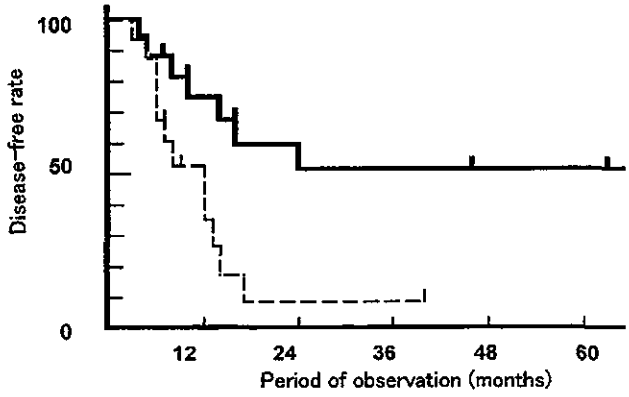

Fig. 1 Disease-free rate curves for 16 patients who underwent adjuvant chemotherapy (–, $n = 16$) and for 15 patients who did not undergo adjuvant chemotherapy (--, $n = 15$) in patients with lymph node metastases ($P = 0.0376$).

cancer is similarly defined as muscle-invasive tumor growth beyond the renal pelvic or ureteral wall and/or regional lymph node metastases (tumor stages pT3, pT4 and/or N+ and M0, 1997 TNM classification).[6] The 5-year relative survival rate for patients with locally advanced bladder cancer has been known to be approximately 50%, and that for patients with advanced upper urinary tract cancer has been known to be at less than 40%.

After the efficacy of combination chemotherapy for metastatic urothelial cancer using MVAC was first described in 1985,[1] several cisplatin-based systemic regimens have been investigated as adjunctive treatments after therapy for locally advanced urothelial cancer by radical surgery.

The German genitourinary oncology group compared cystectomy alone and cystectomy followed by three adjuvant cycles of MVEC (methotrexate, vinblastine, epirubicin and cisplatin) combination therapy in patients with locally advanced bladder cancer.[7] This study demonstrated that there was no disease-specific survival difference, according to the log-rank test. Subgroup analyses also revealed no significant differences for patients with stage pT3pN0, stage pN1 and stage pN2 cancer.[7] Lerner *et al.* retrospectively analyzed long-term progression and survival rates in patients who underwent pelvic lymphadenectomy with en bloc radical cystectomy for bladder cancer and had pathologically proved nodal metastases.[8] Their study showed that there was no significant difference in survival or the interval to progression among patients who received adjuvant chemotherapy compared to those treated with surgery alone.[8]

Skinner and co-workers randomly compared cystectomy plus four cycles of adjuvant chemotherapy (CISCA: cisplatin, cyclophosphamide and adriamycin) and radical cystectomy alone in locally advanced transitional cell carcinoma.[2] The trial demonstrated a significant disease-free survival advantage for the adjuvant treatment arm in the 5 years after cystectomy, whereas overall survival was not significantly prolonged. In a subgroup analysis of patients with only one positive lymph node, patients in the adjuvant treatment arm had superior disease-free and overall survival rates compared to patients in the cystectomy alone arm.[2] Freiha and colleagues compared cystectomy alone and cystectomy followed by adjuvant CMV (cisplatin, methotrexate, vinblastine) combination chemotherapy in patients with locally advanced bladder cancer.[4] The study reported a significant difference in disease-free survival in favor of patients receiving adjuvant chemotherapy, whereas overall survival was not significantly different. Another randomized study by Stockle and colleagues reported adjuvant MVAC or MVEC in 26 of 49 patients with locally advanced bladder cancer.[3] A large, disease-free survival difference at 3.5 years in favor of the 26 patients receiving adjuvant treatment was noted. This study also noted a distinctly improved survival rate for lymph node-positive patients. Of the 13 patients with lymph node-positive status who underwent surgery without adjuvant treatment, 12 developed progressive disease (92%), whereas only three of 11 patients with adjuvant combination therapy developed progressive disease (27%). All three studies have been criticized for their small patient numbers and statistical shortcomings. However, Stockle reported that the addition of 117 non-randomized patients to the original 49 randomized patients (80 patients with three cycles of adjuvant MVAC or MVEC, and 86 patients with cystectomy alone) yielded a highly significant difference in disease-free survival for patients who received adjuvant treatment. This study also demonstrated that adjuvant chemotherapy achieved the highest therapeutic benefit in patients suffering from pN1 disease.[9]

Adjuvant chemotherapy has been a matter of a controversial and serious debate. Improvement of tumor-free and overall survival rates by adjuvant chemotherapy in patients with locally advanced urothelial cancer strongly depends on meticulous selection by the tumor stage. Bono *et al.* randomized only lymph node-negative patients for four courses of adjuvant cisplatin/methotrexate versus cystectomy alone and noted no significant difference in disease-free survival.[10] In the present study, similar to the data reported by Skinner *et al.* and Stockle *et al.*, adjuvant chemotherapy showed a positive impact on disease-free survival in patients with lymph node-positive disease.

In conclusion, our data suggest that adjuvant chemotherapy with MVAC/MEC after radical cystectomy or nephroureterectomy improves disease-free survival rates in patients with pathological lymph node-positive urothelial cancer.

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