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Ⅲ. 研究成果の刊行物・別刷

BJUI
BJU INTERNATIONAL

Dicoumarol enhances doxorubicin-induced cytotoxicity in p53 wild-type urothelial cancer cells through p38 activation

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OBJECTIVE

To investigate the effectiveness of a combined treatment of 3–30-methylene-bis[4-hydroxycoumarin] (dicoumarol) with doxorubicin for the treatment of urothelial cancer, as doxorubicin is a common chemotherapeutic agent but its therapeutic efficacy is limited.

MATERIALS AND METHODS

The synergistic effect of dicoumarol with chemotherapeutic agents such as cisplatin, doxorubicin and paclitaxel was evaluated in RT112 urothelial cancer cells. Then, dicoumarol-mediated enhancement of doxorubicin-induced cytotoxicity was screened in urothelial cancer cell lines with different p53 statuses or RT112 stable transfectants with a dominant-negative mutant of p53 (p53DN). To clarify the importance of the modification of p53 function by dicoumarol to enhance doxorubicin toxicity, the change in the p53-p21 pathway and mitogen-activated protein kinase (MAPK)-mitochondria pathway by the combined treatment were elucidated by Western blot analysis. Finally, the effect of p21 knockdown in the susceptibility to doxorubicin was examined with RT112 stable transfectants with short hairpin RNA (shRNA) of p21.

RESULTS

Dicoumarol significantly increased the susceptibility of RT112 cells to cisplatin and doxorubicin, but not to paclitaxel in RT112 cells. Dicoumarol (100 μM) also enhanced the cytotoxicity of doxorubicin in other bladder cancer cell lines with wild-type p53 (wt-p53; three times in 253J and 13 times in KK47), but not in those with mutant-type p53 (TCCsup, J82 and EJ) or in RT112 p53DN. The combined treatment with dicoumarol suppressed p53/p21 induction by doxorubicin and resulted in sequential p38 MAPK activation, myeloid cell leukaemia 1 suppression and caspase cleavage. The synergistic effect of doxorubicin/dicoumarol was suppressed by the p38 MAPK inhibitor SB202190 and, furthermore, p21 knockdown with shRNA transfection made RT112 cells six times more susceptible to doxorubicin with p38 MAPK activation.

CONCLUSION

These results suggest that concomitant use of dicoumarol could enhance the cytotoxicity of doxorubicin in urothelial cancer cells with wt-p53 through the p53/p21/p38 MAPK pathways. This combined treatment may provide a new therapeutic option to overcome chemoresistance in bladder cancer.

KEYWORDS

urothelial cancer, dicoumarol, doxorubicin, p53, p38

INTRODUCTION

More than 70% of bladder cancers present as moderately-to-well differentiated, superficial papillary TCC, treated with endoscopic transurethral resection of the bladder tumour. Although progression to muscle-invasive cancer is relatively infrequent, >60% of patients have metachronous intravesical recurrence one or more times, and these have a serious impact on patients' quality of life [1]. To prevent intravesical recurrence, prophylactic and therapeutic intravesical instillation of various chemotherapeutic agents has been tried but its efficacy is still limited [2], thus new molecular targets for therapy to enhance its efficacy are being explored.

NADPH: quinone oxidoreductase 1 (NQO1) is a ubiquitous flavoprotein that functions as an antioxidant enzyme [3]. The expression levels of NQO1 are elevated in various cancers compared with surrounding normal tissue [4]. NQO1 was reported to protect cancer cells against anticancer agents through detoxification of intracellular oxidative stress [4]. Furthermore, we recently reported that NQO1 contributed to inhibition of apoptosis through maintenance of p53-p21 expression in urogenital cancer cells with wild-type p53 (wt-p53), and 3–30-methylene-bis[4-hydroxycoumarin] (dicoumarol), which inhibited enzymatic activity of NQO1, promoted cisplatin-induced apoptosis through the attenuation of the p53-p21 pathway [5].

In urothelial cancer, most superficial bladder cancers retain wt-p53 [6], and therefore, we hypothesized that dicoumarol might be appropriate as a modulator of anticancer

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agents for preventing recurrence of superficial bladder cancer and for improving patients' quality of life. In the present study, to assess the potential of dicoumarol in treating superficial bladder cancer cells, we investigated the cytotoxic effects of dicoumarol combined with doxorubicin (a commonly instilled intravesical agent).

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

Antibodies were obtained as follows: anti-p53 (Ab-2) from Calbiochem (San Diego, CA, USA), p21 and NQO1 from Santa Cruz (Santa Cruz, CA, USA), anti-β-actin from Abcam (Cambridge, MA, USA), and antibodies against cleaved poly(ADP-ribose) polymerase (PARP), caspase 8, caspase 9, cleaved caspase 3, c-Jun amino-terminal kinase (JNK), pJNK, pp38, p38, pERK, ERK, pHsp27, Bax, Bcl-2, Bcl-xL and myeloid cell leukaemia 1 (Mcl-1) from Cell Signalling Technology (Beverly, MA, USA). SB202190 as a p38 inhibitor, Z-IETD-FMK as a caspase-8 inhibitor, Z-LEHD-FMK as a caspase-9 inhibitor and Z-VAD(OMe)-FMK as a pan-capase inhibitor were obtained from Calbiochem. Cis-diamminedichloroplatinum (cisplatin) and doxorubicin (adriamycin) were from WAKO (Osaka, Japan), and paclitaxel and dicoumarol were from Sigma (St Louis, MO, USA).

CELL CULTURE

Six urothelial cancer cell lines (RT112, 253J, KK47, TCCsup, EJ and J82) were used in the present study. Sequencing analysis and genotyping of p53 confirmed that RT112, 253J and KK47 had wt-p53 whereas the three other cell lines harboured mutant p53 (mt-p53); J82 harboured double missense mutations (codon 271, CAG→AAG and codon 320, AAG→AAC), TCCsup harboured a nonsense mutation (codon 349, GAA→TAA), and EJ contained a missense mutation (codon 175, CGC→CAC). All six bladder cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

GENOTYPING OF NQO1

DNA was extracted from bladder cancer cells using the QIAamp Blood Kit (QIAGEN, Hilden, Germany). To detect Pro187Ser (C to T at position 609 of the cDNA) NQO1 polymorphism in bladder cancer cell lines, the PCR primers used were 5′-TCCTCAGAGTGGCATTCTGC-3′ and 5′-TCTCCTCATCCTGTACCTCT-3′. PCR conditions were 10 min at 95 °C (one cycle) and 30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C (35 cycles) and 5 min at 72 °C (one cycle). Each PCR product was digested with HinfI for Pro187Ser NQO1 polymorphism. DNA samples were concomitantly amplified and digested together with previously examined DNA samples serving as quality controls. For the Pro187Ser NQO1 polymorphism, restriction fragments were 195 and 35 bp for the Pro allele, and 151, 44, and 35 bp for the Ser allele.

PLASMIDS AND TRANSFECTION

pCMV-neo was a gift from Dr K. Cho (University of Michigan Medical School). Dominant-negative mutant of p53 (p53DN) containing one missense mutation at codon 135 (TGC→TAC), which were purchased from BD Bioscience, were ligated into pCMVneo. psiRNA-h7SKhp21 vectors containing short hairpin RNA (shRNA) targeting p21 were purchased from InvivoGen (San Diego, CA, USA). Cells were transfected with the indicated vectors using Lipofectamine 2000 (Life Technologies, Inc., Grand Island, NY, USA) following the manufacturer's instructions. Stable transfectants were selected by appropriate selection antibiotics, and were confirmed by sequencing, reverse transcription-PCR or Western blot analysis.

CELL VIABILITY ASSAY AND DETECTION OF APOPTOSIS

Cell viability was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described previously [5]. Briefly, 1×10^4 cancer cells with 100 μL suspension were grown in each well of 96-well plates. After 24 h incubation, cells were treated with or without different concentrations of drugs for another 24 h. Then, 20 μL of MTT working solution (5 mg/mL; Sigma) was added to each culture well, and incubated for 4 h. The formazan crystal was dissolved with 100 μL dimethyl sulphoxide. The absorbance (*A*) of each well was measured by a microculture plate reader (Immunoreader; Japan Intermed Co., Ltd, Tokyo, Japan) at 540 nm. The percentage of cytotoxicity = [1 – (*A* of experimental wells/*A* of control wells)] × 100.

To detect the ratio of viable cells or apoptotic cells to total cells, Trypan blue staining or Hoechst 33342 nuclear staining was used. For Trypan blue staining, cells were stained with 0.3% Trypan blue, and counted on haemocytometer. The percentage of viable cells was calculated as the ratio of unstained cells (not blue) to total cells counted. For nuclear staining, cells were stained with 1 mM Hoechst 33342 solution (Wako, Osaka, Japan), and analysed with a fluorescence microscope. Apoptotic cells were identified by morphology and by condensation and fragmentation of their nuclei. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted.

IMMUNOBLOTTING

After drug treatments, cells were washed with PBS and lysed in an appropriate volume of ice-cold RIPA buffer composed of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS containing 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). Cellular lysates were clarified by centrifugation at 13 000*g* for 15 min and the protein concentrations of the lysates were determined by a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of 30–50 μg of the lysates were boiled for 5 min in SDS sample buffer and separated by SDS-PAGE on a 10–15% Tris-HCl minigel, and transferred onto a polyvinylidene difluoride membrane using standard methods. Membranes were probed with appropriate dilutions of primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. After extensive washing, proteins were visualized by a chemiluminescent detection system (GE Healthcare, Buckinghamshire, UK).

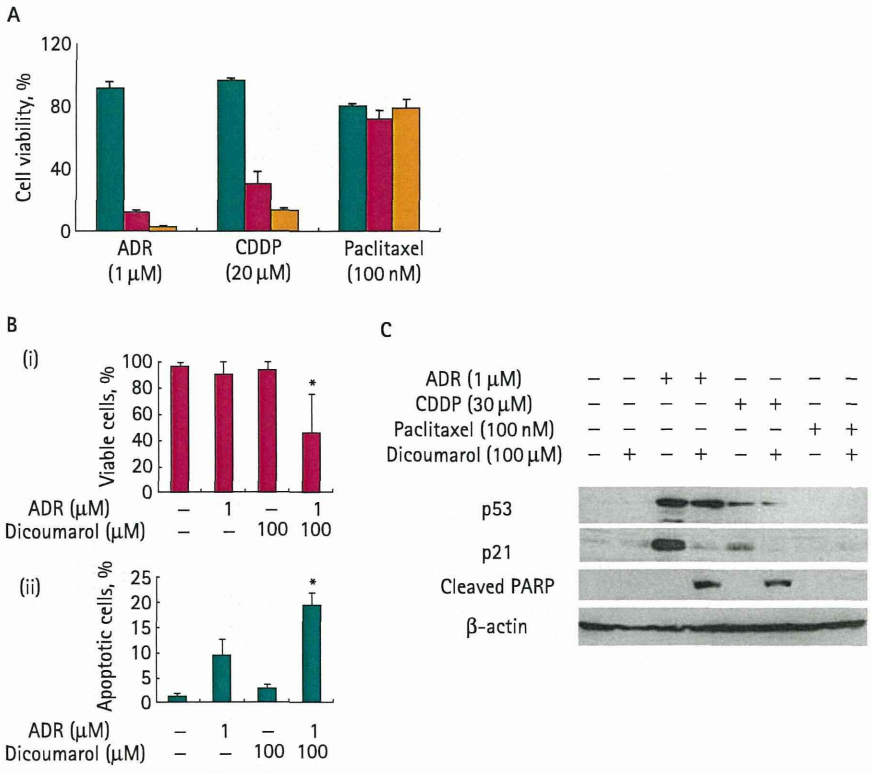
RESULTS

DICOUMAROL ENHANCES DOXORUBICIN- OR CISPLATIN-INDUCED CYTOTOXICITY BUT NOT PACLITAXEL-INDUCED CYTOTOXICITY

We have previously reported that dicoumarol enhanced cisplatin-induced cytotoxicity via suppression of wt-p53 [5]. In the present study, we explored the further potential of dicoumarol to enhance the cytotoxicity of doxorubicin, which is commonly used for treating bladder cancer and paclitaxel, which

is currently investigated for its potential as a therapeutic agent in bladder cancer. In RT112 cells that showed synergism of dicoumarol with cisplatin, cell viability decreased with the combination of dicoumarol with doxorubicin dose-dependently similarly to the case of cisplatin, but there was no effect in the case of paclitaxel (Fig. 1A). To confirm the effect of dicoumarol in enhancing doxorubicin-induced cytotoxicity, Trypan blue staining and Hoechst 33342 nuclear staining were used and showed that the combined treatment decreased cell viability by up to 45% of the control and increased apoptotic cells significantly compared with doxorubicin single treatment ($P < 0.01$). For doxorubicin and cisplatin, dicoumarol suppressed doxorubicin- or cisplatin-induced expression of p53/p21 and evoked PARP cleavage, whereas paclitaxel was unable to induce p53 accumulation and underwent its cytotoxicity through a p53-independent pathway (Fig. 1B). These results suggest that dicoumarol might enhance the cytotoxicity of genotoxic agents that activate the p53 pathway by modulating the interaction between NQO1 and p53.

FIG. 1. The synergistic effect of dicoumarol with chemotherapeutic agents against bladder cancer. ***A****, MTT assay showing the effects of dicoumarol on three kinds of chemotherapeutic agents, cisplatin (CDDP), doxorubicin (ADR) and paclitaxel, in RT112 cells. The results of MTT assays of RT112 treated by various agents at indicated concentrations only (green bar) or with dicoumarol at 100 μM (red bar) or 200 μM (orange bar) for 24 h are shown as the mean ± SD from three independent experiments. Cell viability is indicated by the ratio to cell viability when treated with only the drug vehicle.* ***B****, (i) Viable cell ratio examined by Trypan blue staining showing the combination effect of dicoumarol and ADR in RT112 cells. The results of Trypan blue staining with indicated treatments are shown as the mean ± SD. (ii) Apoptotic cell ratio examined by Hoechst 33342 staining showing the combined effect of dicoumarol and ADR in RT112 cells. The results of Hoechst 33342 staining with indicated treatments are shown as mean values ± SD, *P < 0.001 (two-sided t-tests).* ***C****, Western blot analysis showing the effects of dicoumarol on three kinds of chemotherapeutic agents (CDDP, ADR and paclitaxel) in RT112 cells. RT112 cells were treated with each chemotherapeutic agent for 12 h, with or without 1 h pretreatment with 100 μM dicoumarol, and then underwent Western blot analysis.*



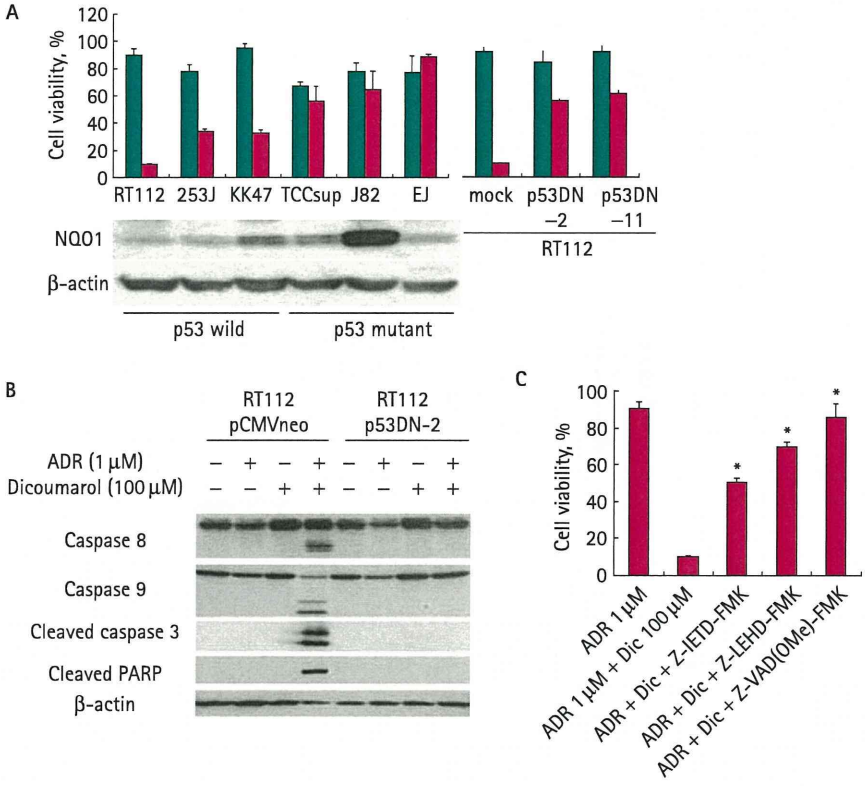
DICOUMAROL PROMOTES DOXORUBICIN-INDUCED APOPTOSIS IN BLADDER CANCER CELLS WITH FUNCTIONAL p53

To examine the importance of functional p53 for dicoumarol to enhance doxorubicin-induced cytotoxicity, the susceptibility of several other bladder cancer cell lines with different p53 statuses to doxorubicin and dicoumarol was monitored by MTT assay. Among the six bladder cancer cell lines, only RT112 cells have heterozygous polymorphism of NQO1, in which C changes to T at position 609 of the cDNA, leading to a change in the amino acid structure of the enzyme. Other cell lines have no polymorphism. All cell lines express NQO1 protein, although RT112 cells expressed relatively low levels of NQO1 protein compared with other cell lines (Fig. 2A). From the MTT assay dicoumarol enhanced doxorubicin-induced cytotoxicity in bladder cancer cell lines with wt-p53 such as RT112, 253J and KK47 (nine times, three times and 13 times against doxorubicin single treatment, respectively), but had no synergistic effect on doxorubicin in three cell lines with mt-p53 (TCCsup, EJ and J82) (Fig. 2A). We could not detect any correlation between NQO1 protein level and the effect of dicoumarol. To elucidate the necessity of functional p53 for synergism, two stable clones transfected with p53DN mutant (RT112 p53DN-2 and -11) were established. Although the antiproliferative effect of dicoumarol itself was not significantly changed by p53DN transfection, MTT assay showed that the effect of dicoumarol in enhancing the susceptibility to doxorubicin was weakened in both p53DN transfectants (Fig. 2A). Also, using Western blot analysis, the combination of doxorubicin and dicoumarol activated caspases in RT112 pCMVneo but not in RT112 p53DN-11 (Fig. 2B), and the inhibition of caspase activity by specific caspase inhibitors in RT112 pCMVneo suppressed the synergistic effect of dicoumarol (Fig. 2C). These results indicate that functional p53 is necessary for dicoumarol to enhance doxorubicin-induced apoptosis in urothelial cancer cells.

DICOUMAROL ENHANCED DOXORUBICIN-INDUCED CYTOTOXICITY VIA ACTIVATION OF THE p38 SIGNALLING PATHWAY IN BLADDER CANCER WITH wt-p53

The activation of mitogen-activated protein kinase (MAPK) cascades is considered to be one of the mechanisms by which chemotherapeutic agents induce apoptosis [7,8]. Whereas we have previously reported that dicoumarol activated the JNK pathway when used with cisplatin [5], the influence of dicoumarol on MAPK cascades when used with doxorubicin was investigated in the

FIG. 2. The acceleration of doxorubicin (ADR)-induced apoptosis by dicoumarol in urothelial cancer cells with wt-p53 through a caspase-dependent pathway. ***A****, The effects of dicoumarol on ADR-induced cytotoxicity assessed by the status of p53 and NQO1, using six urothelial cancer cell lines and two stable clones of RT112 transfected with p53 dominant-negative mutants (RT112 p53DN-2 and RT112 p53DN-11). The results of MTT assays with 1 μM ADR with or without 100 μM dicoumarol are shown as mean values ± SD from three independent experiments.* ***B****, Comparison of the response to ADR and dicoumarol between RT112 control transfectants (RT112 mock) and p53DN transfectant (RT112 p53DN-11), shown by Western blot analysis. Cells were treated with 1 μM ADR for 12 h, with or without 1 h pretreatment of 100 μM dicoumarol, and then lysed in RIPA buffer and subjected to Western blot analysis.* ***C****, The inhibiting effect of caspase inhibitors on dicoumarol-mediated enhancement of ADR-induced cytotoxicity in RT112 cells. Cells were pretreated with or without 100 μM dicoumarol for 1 h, followed by treatment with either ADR alone or ADR plus 80 μM Z-IETD-FMK (caspase-8 inhibitor), 100 μM Z-LEHD-FMK (caspase-9 inhibitor) or Z-VAD(OMe)-FMK (pan-caspase inhibitor), respectively, for an additional 24 h. Cell viability is indicated by the ratio to cell viability when treated with ADR alone. *P < 0.01 (two-sided t-test) vs ADR/dicoumarol combined therapy.*



present study. The doxorubicin/dicoumarol combined treatment attenuated p53/p21 induction in the same manner as the cisplatin/dicoumarol combination. Among MAPK pathways, phosphorylation of p38 was more strongly induced 9 h after the treatment, although the expression levels of p38 were not changed (Fig. 3A).

To investigate the significance of p38 activation in the present experiments, RT112 cells were treated with SB202190, a specific inhibitor of p38. Although SB202190 (30 μM) alone slightly increased the cytotoxicity of doxorubicin, pretreatment with SB202190 apparently attenuated the effects of dicoumarol to enhance doxorubicin-induced cytotoxicity (Fig. 3B). On the other hand, SP600125, a specific JNK inhibitor, did not suppress apoptosis induced by doxorubicin/dicoumarol combined treatment (data not shown). The doxorubicin/dicoumarol combined treatment did not change the expression of Bax, Bcl-2 or Bcl-xL, but suppressed the expression of Mcl-1, which is one of the anti-apoptotic BH3 proteins. The inhibition of p38 activation by SB202190 restored Mcl-1 expression, and suppressed activation of caspase 3 (Fig. 3B). These findings suggest that dicoumarol enhanced the cytotoxicity of doxorubicin via activation of the p38 signalling pathway.

p21 ATTENUATION BY DICOUMAROL CONTRIBUTED TO PROMOTE p38 ACTIVATION IN UROTHELIAL CANCER CELLS WITH wt-p53

We examined the relationship between p53 status and the effect of dicoumarol to activate p38. The change of p38 status was examined in RT112 p53DN or other urothelial cancer cell lines after each drug exposure. The suppression of p53 function by p53DN attenuated p38 activation in RT112 cells (Fig. 4A). There were similar tendencies in 253J cells that retain wt-p53 and J82 cells harbouring mt-p53 (Fig. 4B). In 253J cells, dicoumarol/doxorubicin combined treatment attenuated p53/p21 expression and induced p38 phosphorylation followed by apoptotic change. In contrast, in J82 cells, the normal function of the p53 gene was lost. There was no p21 induction after doxorubicin exposure, and dicoumarol/doxorubicin combined treatment did not induce the phosphorylation of p38. These results indicate that dicoumarol could evoke p38 activation only in cancer cells with wt-p53.

To confirm whether the suppression of p21 expression by dicoumarol contributed to enhance doxorubicin-induced cytotoxicity via p38 activation, we established a stable clone of RT112 transfected with a p21siRNA vector (RT112 p21siRNA-8) and investigated the p38 status and susceptibility to doxorubicin. The suppression of p21 expression made RT112 cells about six times more susceptible to doxorubicin in MTT assay and, at that time, the p38 signalling pathway was activated and Mcl-1 expression was suppressed (Fig. 4C). All together, these results indicate that p21 attenuation by dicoumarol contributed to p38 activation and enhancement of doxorubicin-induced cytotoxicity in urothelial cancer cells with wt-p53.

DISCUSSION

Due to its up-regulation in tumour lesions, NQO1 has been considered a good molecular target in bladder cancer. Mitomycin C and its structurally related compound EO9, which are activated by NQO1, have shown their effectiveness as intravesically instilled agents, although the relationship between the response and NQO1 is still controversial [9,10]. In the present study, we used dicoumarol, an inhibitor of the enzymatic activity of NQO1,

and showed that it contributed to enhance doxorubicin-induced cytotoxicity through p38 activation in urothelial cancer cells with wt-p53.

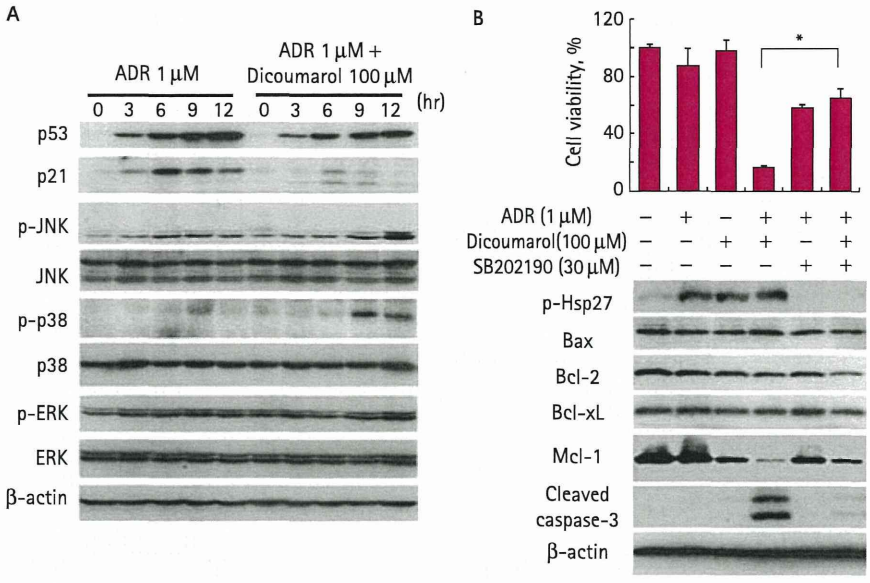
NQO1 has a function to stabilize p53 protein. NQO1 inhibition by dicoumarol therefore induced p53 degradation and was reported to block wt-p53-mediated apoptosis induced by γ-irradiation [11]. However, p53-dependent p21 induction has recently been considered to inhibit the apoptotic response, and p21 attenuation has the potential to make genotoxic chemotherapeutic agents more effective [12,13]. Although p53 mutation has an important role in bladder cancer invasion and progression, most low to intermediate risk superficial bladder cancers retain wt-p53 and intravesical treatment with genotoxic agents such as doxorubicin could not show sufficient effectiveness. If this resistance against doxorubicin may even partially come from chemoresistant mechanism of p53-p21 pathway, we think that the combination of dicoumarol can be useful to enhance its effect in bladder cancer.

The present results showed that the combination of dicoumarol with genotoxic agents suppresses p21 induction by functional p53 and simultaneously evokes the stress-activated protein kinase (SAPK) family such as JNK and p38 signalling pathways, resulting in the induction of apoptosis. We think that mechanisms between p21 suppression and the activation of SAPK family should be further elucidated in various cell lines, because p53 mutant cell lines such as RT112 p53DN and J82 did not show p38 activation after doxorubicin or doxorubicin/dicoumarol treatment although they had already lost p21 inducibility. Concerning the anticancer effect of dicoumarol, several reports have referred to its effect to induce oxidative stress [14,15], and intracellular reactive oxygen species status is also important to activate SAPK family [16]. We now speculate that p21 attenuation is one of the mechanisms of dicoumarol to activate SAPK family but other additional effects of dicoumarol are also necessary to activate SAPK family.

The present results showed the possibility that the Mcl-1 protein may be a downstream molecule regulated by the p38 signalling pathway. Mcl-1 decreased when apoptosis was induced with p38 activation, and inhibition of p38 activation by SB202190 restored the Mcl-1 protein level. Mcl-1 is an anti-apoptotic Bcl-2 family member. Several studies have shown that Mcl-1 plays a particularly important functional role in promoting survival in malignant haematopoietic cells, including leukaemia and myeloma cells, although there has been no report on its role in urothelial cancer [17,18]. Recently, the possibility was suggested that p38 MAPK may positively or negatively regulate Mcl-1 stability and turnover [19], and therefore, we propose that the suppression of Mcl-1 protein by p38 activation may have a crucial role in the doxorubicin/dicoumarol combined treatment. We would like to further elucidate the importance of this pathway in the apoptotic process of urothelial cancer in the future.

In conclusion, in the present study dicoumarol sensitized urothelial cancer cells with wt-p53 to doxorubicin through p38 activation induced by the suppression of the p53/p21 pathway. Most low to intermediate risk superficial bladder cancers retain wt-p53, and we think that the present results will provide a new strategy to overcome rapid or frequent recurrence of such superficial bladder cancers after conventional intravesical instillation therapy. In those cancers with wt-p53, we think that the risk of transient combined therapy leading to selective progression of p53-mutant cancer cells may be low, although we have to pay attention to this possibility in clinical setting. Furthermore, because dicoumarol enhanced cisplatin- and doxorubicin-induced cytotoxicity through different MAPK cascades, a concomitant use of dicoumarol may effectively enhance the cytotoxicity of conventional multi-drug combined chemotherapy such as methotrexate, vinblastine, doxorubicin and cisplatin (MVAC), and cisplatin, methotrexate and vinblastine (CMV) for invasive TCC. We are planning to start the evaluation of its efficacy and safety in an *in vivo* orthotopic or metastatic mouse model.

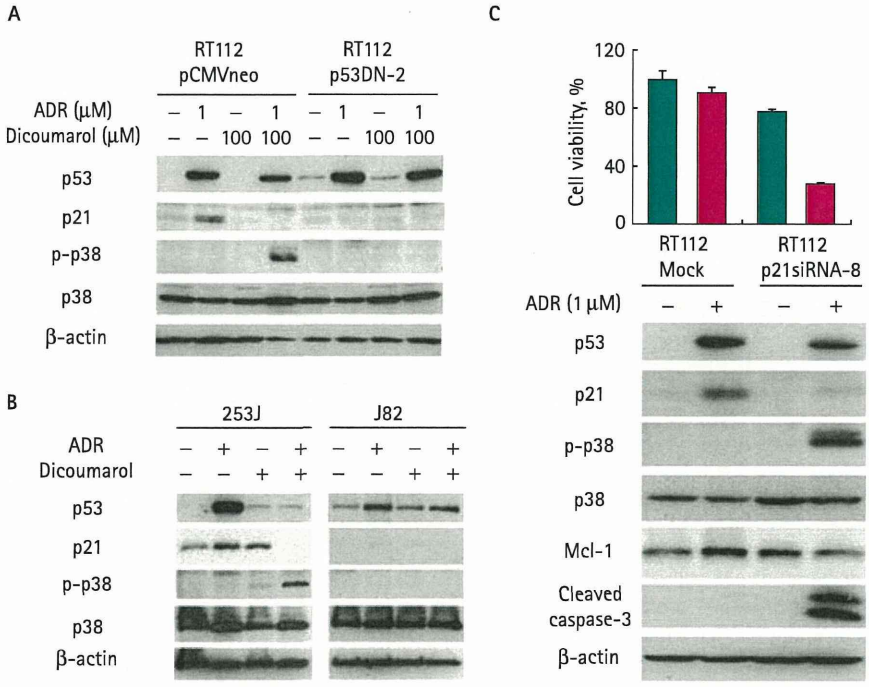
*FIG. 3. Dicoumarol promotes doxorubicin (ADR)-induced apoptosis via activation of the p38 signalling pathway. **A**, Western blot analysis showing changes of the p53-p21 pathway and the MAPK cascade in RT112 treated with ADR with or without dicoumarol. Cells were treated with 1 μM ADR for 12 h, with or without 1 h pretreatment of 100 μM dicoumarol. '0 h' refers to the time of ADR addition. **B**, MTT assay (upper panel) and Western blot analysis (lower panel) showing the effect of inhibition of the p38 signalling pathway on dicoumarol-mediated enhancement of ADR-induced apoptosis in RT112. MTT assays with 1 μM ADR with or without 100 μM dicoumarol and/or 30 μM SB202190 are shown as mean values ± SD from three independent experiments. *P < 0.01 (two-sided t-test) vs ADR/dicoumarol and ADR/dicoumarol/SB202190. The status of molecules associated with apoptosis in RT112 treated with ADR, dicoumarol and SP202190, a p38 specific inhibitor, are shown in Western blot analysis after cells were treated with the indicated drugs for 12 h.*



CONFLICT OF INTEREST

None declared.

*FIG. 4. Dicoumarol activates the p38 signalling pathway in p53 wt-cells via p21 attenuation. **A**, Comparison of p38 status between RT112 control transfectants (RT112 mock) and p53DN transfectant (RT112 p53DN-11) treated with doxorubicin (ADR) and dicoumarol, shown by Western blot analysis. Cells were treated with 1 μM ADR for 12 h, with or without 1 h pretreatment of 100 μM dicoumarol, and then lysed in RIPA buffer and subjected to Western blot analysis. **B**, Western blot analysis showing changes in the p53-p21 pathway and p38 phosphorylation in 253J cells (wt-p53) and J82 cells (mt-p53) treated with ADR with or without dicoumarol. Cells were treated with the indicated drugs for 12 h, and then lysed in RIPA buffer and subjected to Western blot analysis. **C**, MTT assay (upper panel) and Western blot analysis (lower panel) showing the induction of p38 activation and apoptosis by the attenuation of p21 expression. RT112 cells stably transfected with control vector (RT112 mock) or p21siRNA vector (RT112 p21siRNA-8) were treated with 1 μM ADR for 12 h, and then p53 and p21 expression, p38 phosphorylation, Mcl-1 and cleaved caspase 3 were examined in Western blot analysis. Cell viability was examined by MTT assay 24 h after the treatment.*



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Abbreviations: **(MA)(SA)PK**, (mitogen-activated) (stress-activated) protein kinase; **p53DN**, dominant-negative mutant of p53; **shRNA**, short hairpin RNA; **Mcl-1**, myeloid cell leukaemia 1; **NQO1**, NADPH: quinone oxidoreductase 1; **(wt)(mt)-p53**, (wild-type) (mutant) p53; **MTT**, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay; **PARP**, poly(ADP-ribose) polymerase; **JNK**, c-Jun amino-terminal kinase.

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Is T1G3 Bladder Cancer Having a Definite Muscle Layer in TUR Specimens a Highly Progressive Disease?

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Objective: Patients with T1G3 bladder cancer are at high risk of progression to muscle-invasive cancer, and early cystectomy is considered as a treatment option in this particular situation. On the other hand, understaging of T1G3 bladder cancer has been gradually proven as second or repeat transurethral resection (TUR) has been widely applied. To evaluate the real rate of progression, we investigated the prognosis of T1G3 bladder cancer in which a muscle layer was histologically confirmed in the TUR specimens.
Methods: We retrospectively reviewed 48 patients with primary T1G3 bladder cancer in which a muscle layer in the TUR specimens was confirmed between 1990 and 2006 in our institute. We investigated recurrence and progression in 45 patients, excluding 3 who were immediately treated with radical cystectomy. Fifteen and 12 patients received intravesical treatment with bacillus Calmette–Guérin (BCG) and anticancer agents just after TUR, respectively. The remaining 18 did not have any such treatment.
Results: Recurrence and progression were observed in 21 (47%) and 3 patients (6.7%), respectively, during a median follow-up period of 42.1 months. The 3-year recurrence-free and progression-free survival rates were 54% and 91%, respectively. No significant differences were observed in the rates between the patients with and without BCG treatment in the study.
Conclusions: There is a possibility that the progression rate in patients with T1G3 bladder cancer is not as high as previously reported when only patients whose muscle layer was histologically confirmed were analyzed. An adequate technique for TUR that unmistakably collects the muscle layer may be important to predict the outcome accurately.

Key words: T1G3 – bladder cancer – progression – understaging – muscle layer

INTRODUCTION

Approximately 70% of bladder tumors are diagnosed as superficial cancer without invasion to the muscle layer at initial presentation (1). Although the prognosis of superficial bladder cancer is generally favorable, it is known that T1G3 bladder cancer is a distinct clinical entity from the remaining superficial cancer since it has been reported that the disease eventually progresses in approximately half of the patients with T1G3 cancer during follow-up (2,3). Because of its high risk for progression, early radical cystectomy is sometimes considered as an initial treatment, although it is controversial (4).

As second or repeat transurethral resection (TUR) for T1 bladder cancer is becoming widely used, it is known that understaging of T1 cancer frequently occurs (5). Although T1 cancer is defined as a tumor with invasion to the lamina propria but no invasion to the detrusor muscle, it seems to be clinically composed of two types. One is genuine T1 cancer in which cancer cells really exist up to the lamina propria. The other is T2 or higher cancer misdiagnosed as T1 cancer because the detrusor muscle is not included in the TUR specimens.

As previously noted, it has been believed that patients with T1G3 cancer have a high progression rate. If there is contamination by T2 or higher cancer, the prognosis of T1G3 cancer must be worse than the true one. However, only a few reports analyzing the prognosis of T1G3 cancer demonstrate whether the TUR specimens included the

detrusor muscle (6). In the present study, we retrospectively evaluated recurrence and progression in patients with T1G3 bladder cancer whose detrusor muscle was histologically confirmed.

PATIENTS AND METHODS

In our institute, TUR of bladder cancer was done in the standard manner. Once all visible tumors were resected, the base of the main tumor was resected again until the perivesical fat tissue became visible. Care had to be taken not to extensively penetrate the bladder wall. Between 1990 and 2006, we treated 48 patients with initially diagnosed T1G3 bladder cancer in our institute. It was histologically confirmed that TUR specimens derived from all patients included enough of the muscle layer to adequately determine the T stage. On the other hand, there were another 11 patients diagnosed with T1G3 bladder cancer at the same period in our institute who had no evaluable muscle layer in the TUR specimens. Patients who had findings suggestive of muscle-invasive bladder cancer on radiographic studies and a history of upper urinary tract cancer were excluded. In addition, patients who did not receive regular cystoscopic examination every 3 months after TUR were excluded.

Of the 48 patients, 3 patients underwent immediate radical cystectomy because of the patients' decisions, based on concomitant localized prostate cancer, severe hematuria and storage symptoms caused by bladder cancer. In the remaining 45 patients with T1G3 cancer, the bladder was preserved. Eighteen patients did not have any bladder instillation therapy after TUR. Of the remaining 27 patients, 15 and 12 received intravesical treatment with bacillus Calmette–Guérin (BCG, 80 mg of Tokyo strain for 8 patients and 81 mg of Connaught strain for 7 patients) and an anticancer agent (anthracyclines for 8 patients and mitomycin C for 4 patients), respectively. BCG was intravesically instillated every week for 6–8 weeks as one course and every patient received at least one course of BCG. None received maintenance BCG therapy. Because of the retrospective nature of the present study, indications for additional therapy and criteria for drug selection were not uniform. However, patients who had carcinoma *in situ* or persistent positive urine cytology after TUR were exclusively treated with BCG (Table 1).

The patients were followed by cystoscopic examination and urine cytology every 3 months. If necessary for suspicious symptoms and findings of metastatic development, radiographic examination using computed tomography and chest X-rays was conducted. When bladder tumors were found on cystoscopic examination, TUR was performed to pathologically confirm recurrence. Invasion of the muscle layer, as well as development of distant metastasis, was defined as progression.

The Kaplan–Meier method and log-rank test were used for statistical analysis of recurrence and progression. A value of $P < 0.05$ was defined as statistically significant.

Table 1. Backgrounds of patients treated with BCG and an anticancer agent

	BCG ($n = 15$)	Non-BCG ($n = 30$)
Carcinoma *in situ*	3 (20.0%)	0 (0%)
Positive cytology[a] (after TUR)	2 (13.3%)	0 (0%)
Tumor size (mm)		
<10	6 (40.0%)	4 (13.3%)
≥10	9 (60.0%)	26 (86.7%)
Tumor number		
Solitary	3 (20.0%)	9 (30.0%)
Multiple	12 (80.0%)	21 (70.0%)
Repeat TUR	1 (6.7%)	0 (0%)

BCG, bacillus Calmette–Guérin; TUR, transurethral resection.
[a]Patients who represented positive urine cytology after TUR were different from patients who had carcinoma *in situ*.

RESULTS

The median age of the 45 patients (42 men and 3 women) at initial TUR was 68 years, ranging from 29 to 97. The median follow-up period was 42.1 months (range, 9.5–131.4).

Recurrence was observed in 21 (47%) patients. The recurrence-free survival rates at 2 and 3 years were 57% and 54%, respectively (Fig. 1). Most recurrences occurred within 2 years after TUR. The recurrence-free survival rate in the 15 patients treated with BCG was 73% at 2 and 3 years (Fig. 2). Although the 50% 2-year and 44% 3-year recurrence-free survival rates in 30 patients without BCG were lower than those with BCG, there was no significant difference in recurrence-free survival between them ($P = 0.097$, log-rank test). The 3-year progression-free survival rate of the 45 patients was 91% (Fig. 3).

Progression was observed in three patients (6.7%). None of the three patients received BCG therapy or had distant metastasis at the initial presentation of progression. After progression, two patients (4.4%) eventually died of bladder cancer.

Pathological comparison between the TUR and cystectomy specimens in the three patients treated with immediate radical cystectomy revealed understaging for one patient (pT3pN0). The patient developed distant metastasis 19 months after surgery and finally died of bladder cancer in spite of treatment by several courses of systemic chemotherapy.

Of the 11 patients without a muscle layer in the TUR specimens, 6 (54.5%) and 3 (27.3%) patients showed recurrence and progression, respectively. Eventually, two patients died of bladder cancer.

DISCUSSION

One of the biggest problems of T1G3 bladder cancer is progression to muscle-invasive cancer. Since it is obvious that

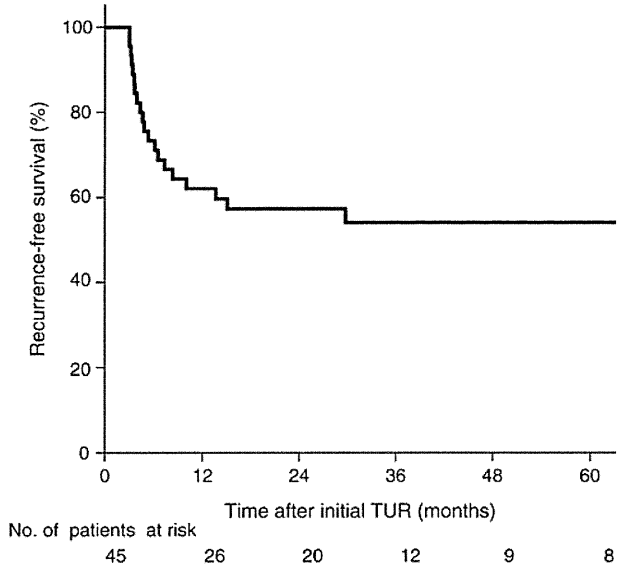

Figure 1. Recurrence-free survival in 45 patients with primary T1G3 bladder cancer for which the muscle layer was histologically confirmed. TUR, transurethral resection.

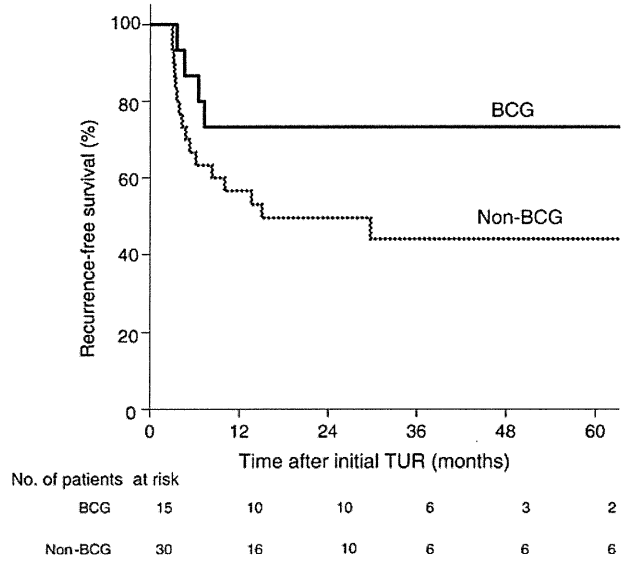

Figure 2. Recurrence-free survival in patients with BCG ($n = 15$) and without BCG treatment ($n = 30$). No significant difference was observed ($P = 0.097$, log-rank test). BCG, bacillus Calmette–Guérin.

the prognosis of muscle-invasive cancer is not promising, to accurately predict the probability of progression in patients with T1G3 cancer is clinically important to make treatment decisions, especially considering whether immediate radical cystectomy should be offered as the initial treatment.

Previous studies have demonstrated that the recurrence and progression rates in patients with T1G3 cancer vary widely from 27% to 70% and 4% to 33%, respectively (Table 2) (3,6,13,14). Such variation may indicate that T1G3 cancer is composed of heterogeneous groups. Since it is known that understaging of T1 cancers frequently occurs (4), the degree of contamination by muscle-invasive cancer in

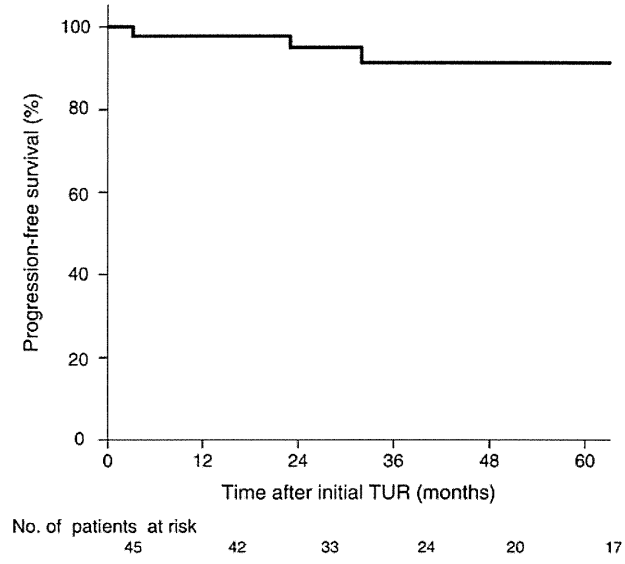

Figure 3. Progression-free survival in 45 patients with primary T1G3 bladder cancer for which the muscle layer was histologically confirmed.

Table 2. Recurrence and progression rates of T1G3 bladder cancer

Author (reference)	No. of patients	Median follow-up period (months)	Recurrence rate (%)	Progression rate (%)
Gohji et al. (6)[a]	45	63	36	4
Brake et al. (13)	44	43	27	16
Patard et al. (14)	50	65	52	22
Shahin et al. (3)	92	64	70	33
Present study[a]	45	42	47	6.7

[a]Studies analyzed patients whose muscle layers were histologically included in the transurethral specimens.

T1G3 cancer may be related to the differences in progression rates among the reports.

Dutta et al. (7) performed radical cystectomy for 63 patients who were diagnosed as having T1 bladder cancer. The muscle layer was confirmed in the TUR specimens from 37 patients, whereas it was not included for 26 patients. Pathological evaluation of cystectomy specimens indicated that 30% and 62% of the patients with and without the muscle layer in the TUR specimens showed pT2 or higher diseases, respectively.

Herr (5) reported a similar result. They performed repeat TUR for 58 patients who were considered to have T1 bladder cancer in the initial TUR. Repeat TUR found muscle invasion in 5 patients (14%) with the muscle layer and in 11 patients (49%) without the muscle layer in the initial TUR. Thus, it is apparent that understaging of T1 cancer is likely to occur if the muscle layer is not included in the TUR specimen.

In the present study, the progression rate of T1G3 bladder cancer during 42 months of follow-up was 6.7% when only

patients with a histologically confirmed muscle layer in the TUR specimens were analyzed. The 3-year progression-free survival rate was 91%. On the other hand, the 27.3% of the progression rate in the patients without a muscle layer was much higher, although it is hard to compare the rates of the two groups because of the small number of the patients. Gohji et al. (6) reported the lowest progression rate to our knowledge (Table 2). Their study consisted of 45 patients with T1G3 cancer whose TUR specimens included a definite muscle layer. Thus, the progression rate in patients with T1G3 cancer was not so high if we analyzed only patients whose muscle layer was histologically confirmed, which suggests less possibility of contamination by muscle-invasive bladder cancer. In the present study, of the three patients treated with immediate radical cystectomy, one was proven to be understaged because muscle invasion was found in the cystectomy specimen. If this patient is followed without immediate radical cystectomy, progression should be observed. However, even though we assume the patient to be a subject with progression, the progression rate, 8.7% (4 of the 46 patients), was still lower than those in the previous reports, although it is hard to draw a solid conclusion because of the small sample size in this study.

The second clinical problem of T1G3 cancer is its high recurrence rate. Similar to the previous reports, recurrence was observed in 47% in the present study (Table 2). Recurrence occurred within 2 years in most patients. To prevent early recurrence in T1G3 cancer, BCG or anticancer agents are indicated (8,9). Although there was no significant difference in the recurrence-free survival rate between patients with and without BCG therapy in our study, the lack of uniform criteria for intravesical therapy and small sample size may have influenced the result. There was a 29% difference at 3 years after TUR between the two groups and no recurrence was observed after 1 year in the BCG group. Several large studies have indicated that intravesical BCG therapy makes a contribution to prevent recurrence for patients with superficial bladder cancer superior to those of intravesical anticancer agents (10–12).

As previously discussed, it is likely that understaging is more frequent in T1G3 bladder cancer in which the muscle layer is not included. On the other hand, understaging was sometimes observed even in patients whose muscle layer was sufficiently collected in the TUR specimens. In the present study, one of the three patients with muscle-layer-confirmed T1G3 who underwent immediate cystectomy had pT3 bladder cancer. Herr (5) reported that repeat TUR for 35 patients with T1 bladder cancer having a definite muscle layer in the initial TUR showed residual cancer in 26 (74%) and muscle-invasive cancer in 5 (14%). A recent randomized study done by Divrik et al. (15) indicated that T1 patients with repeat TUR plus intravesical mitomycin C had significantly lower recurrence (26%) than those with only initial TUR plus intravesical mitomycin C (63%). In addition, the progression rate was lower in patients with repeat TUR (4%) than in those without it (12%), although the difference did not reach statistical significance ($P = 0.097$). Thus, repeat TUR may contribute to not only more accurate diagnosis and a more precise prognosis but also an improved outcome for patients with T1G3 cancer whose muscle layer is confirmed in the initial TUR specimen.

In conclusion, there is a possibility that the progression rate in patients with T1G3 bladder cancer is not as high as previously reported when only patients whose muscle layer was histologically confirmed were analyzed. An adequate technique for TUR that clearly collects the muscle layer may be important to predict the outcome accurately.

Conflict of interest statement

None declared.

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Bladder Cancer Working Group Report

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Epidemiology of bladder cancer: Bladder cancer is the 7th most common cancer in men and the 17th most common in women in the world. The incidence of bladder cancer varies considerably among countries, with the highest incidence rates seen in Western countries and the lowest rates in Asian countries. In recent years, the mortality rate due to bladder cancer has been stable or decreased gradually. Lifestyle and urothelial carcinoma: Occupational risks, environmental risks, dietary habits and cigarette smoking are lifestyle factors known to influence the development of urothelial carcinoma. Although the relative risk of bladder cancer associated with occupations is small, the public health impact may be significant. The Western pattern of diet is associated with a significant increase in the risk of bladder cancer. It has been found that smoking accounts for more than 50% of bladder cancers in men and 30% in women. Urological patients' awareness of smoking as a risk factor for bladder cancer is lower than their awareness regarding other smoking-related disease entities. Counseling patients regarding the risk of tobacco is a role for urologists. Genetic susceptibility to urothelial carcinoma: Recent single-nucleotide polymorphism genetic studies in relation to bladder carcinogenesis have revealed several associated genetic polymorphisms of detoxification or DNA repair genes, such as NAT2, GST and OGG1. That information is important in relation to environmental risk factors and ethnic differences and will help predict the prognosis of patients with bladder cancer. Further studies are needed to confirm potential gene–gene and gene–environmental interactions leading to bladder carcinogenesis.

Key words: bladder cancer – epidemiology – risk factors

The Working Report Urological Cancer: Bladder cancer presentation was divided into three chapters: the epidemiology of bladder cancer, lifestyle and urothelial carcinoma, and genetic susceptibility to urothelial carcinoma.

EPIDEMIOLOGY OF BLADDER CANCER

Statistics regarding the epidemiology of bladder cancer show that about 357 000 new cases of bladder cancer were diagnosed in the world in 2002 (Table 1). This is the 7th most common cancer in men and the 17th most common in women. Bladder cancer is three to four times more common in men than in women. Bladder cancer has a higher incidence in the USA and Europe compared with Asian countries. Regarding the mortality of bladder cancer, in 2002, about 145 000 patients died in the world. The age-standardized mortality rates are 2–10 per 100 000 males and 0.5–4 per 100 000 females (1).

Looking at various countries, the age-standardized incidence rate for bladder cancer in 2005 was highest in Italy, whereas Japan, China and India had the lowest rates shown in Fig. 1 (1). American whites and blacks showed very different rates. In the most recent data, for 2008, the incidences had gone up, but not greatly. Spain became the top country, followed by Italy. African-Americans again had a lower incidence of bladder cancer than white Americans, but it is interesting that they had a higher mortality rate (2).

Table 1. Epidemiology of bladder cancer

357,000 new cases in the world (2002)
7th most common cancer in males
17th in females
3 to 4 times more common among males than among females
USA and European countries > Asian countries
Age-adjusted mortality
2-10 per 100,000 males
0.5-4 per 100,000 females

Regarding the distribution of tumor histology in black and white patients in the USA, almost all whites had pure urothelial carcinoma, whereas only about 80% of blacks did. The other histologies were about the same. The distribution of the T stage shows that 81% of whites had non-muscle invasive bladder cancer, compared with 62% of blacks. This is a very different distribution. A significant difference in survival was seen for T3 patients by race (3).

The incidence of bladder cancer in Japanese males in 2002 was about 20 per 100 000 population. Similarly, for Japanese women, the incidence of bladder cancer is <5 per 100 000 population. The trends in the crude cancer incidence rate per 100 000 Japanese population show that bladder cancer is increasing in both Japanese men and women. However, the age-standardized bladder cancer incidence rates per 100 000 world population have not been increasing much and are almost stable. Since 1980, malignant neoplasms have shown the highest mortality rate among diseases in Japan. The mortality rates for bladder cancer have increased in both Japanese males and females. However, the increase has not been very great in the case of the age-standardized bladder cancer death rates, and the rate actually decreased in females (Fig. 2). The mortality rate in Japanese males in 2006 was about 6 deaths per 100 000, whereas it was about 3 or 4 deaths per 100 000 females. The crude cancer mortality rates per 100 000 Japanese population for each sex have gradually increased. However, the age-standardized bladder cancer death rates per 100 000 world population have remained steady for both genders (http://www.ncc.go.jp/index.html).

In international comparison, the proportions of deaths of males due to bladder cancer are about 5% in Japan and Korea, and about 6.5% in the USA. On the other hand, the rates for females are about 2% in Japan and Korea, and about 3% in the USA. The age-standardized bladder cancer death rates per 100 000 Japanese 1985 standard population show that there has generally been stability during the last 40 years in both males and females. The age-standardized bladder cancer mortality rates for elderly males have increased, but they have been stable for middle-aged male patients. Females showed the same tendency (http://www.ncc.go.jp/index.html).

About 6000 bladder cancer patients were registered by the Japanese Urological Association during the 3 years from 1999 through 2001 (Fig. 3). On the basis of the T staging, about 73% of those patients had non-muscle invasive bladder carcinoma and 23% had invasive disease, for a ratio of about 3 to 1. In the clinical N stage and M stage findings, 81% were N0 and 83% were M0. The predominant histology was urothelial carcinoma, i.e. transitional cell carcinoma, seen in 94% of patients. The highest grade and predominant grade data showed that close to half of the Japanese bladder cancer patients were G2. The pathological stage data showed that 75% were pTis, pTa or pT1, and 18% were muscle-invasive pT2 through pT4. Regarding the mode of treatment of those patients, nearly 80% underwent transurethral surgery (TUR/TUC), and close to 20% underwent total cystectomy (4).

In conclusion, the incidence of bladder cancer varies considerably among countries, with the highest incidence rates seen in Western countries. In recent years, the mortality rate due to bladder cancer has been stable or decreased gradually.

LIFESTYLE AND UROTHELIAL CARCINOMA

Occupational risks, environmental risks, dietary habits and cigarette smoking are lifestyle factors known to influence the development of urothelial carcinoma. As occupations at risk for urothelial and bladder cancers, the recent literature points out bitumen (asphalt, tar) workers, automobile industry workers, hairdressers using colorants, sewing machine workers, painters, printers and paperhangers, and truck and bus drivers (Table 2). Regarding exposure to colorants, or dyes, occupational exposure to aromatic amines is a known bladder cancer risk factor. However, the impact of exposure to azo dyes, which may release aromatic amines in humans, is at present controversial.

A German case-controlled study was published in 2008 that investigated 156 bladder cancer patients and 336 control subjects (5). The odds ratio for painters was reported to be 1.98, with a 95% confidence interval (CI) of 0.64–6.11. For hairdressers, the odds ratio was 4.9 (95% CI: 0.85–28.39). For the wood processing occupation, the odds ratio was 1.19 (95% CI: 0.58–2.41), and for chronic exposure to colorants, the odds ratio was 1.84 (95% CI: 0.68–4.95). Individuals exposed to colorants showed an elevated risk for bladder cancer, but the significance was borderline.

The International Agency for Research on Cancer submitted a report in 2008 in Lyons, France. They reported that 80% of modern hair dyes are permanent (oxidative) hair dyes and consist of colorless primary intermediates and couplers. They also noted that current epidemiological studies have shown a small but consistent increase in bladder cancer in male hairdressers and barbers. They concluded that hair dyes are probably a Grade 2A carcinogen in humans.

A 2009 report dealt with a study of the risk of bladder cancer in the automobile industry that was conducted as a

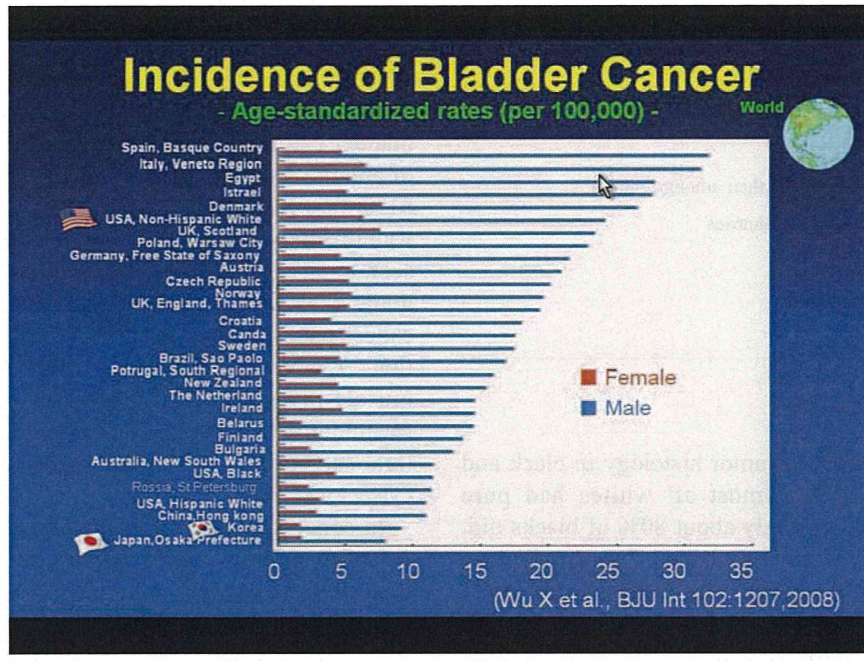

Figure 1. Incidence of bladder cancer: age-standardized rates (per 100 000).

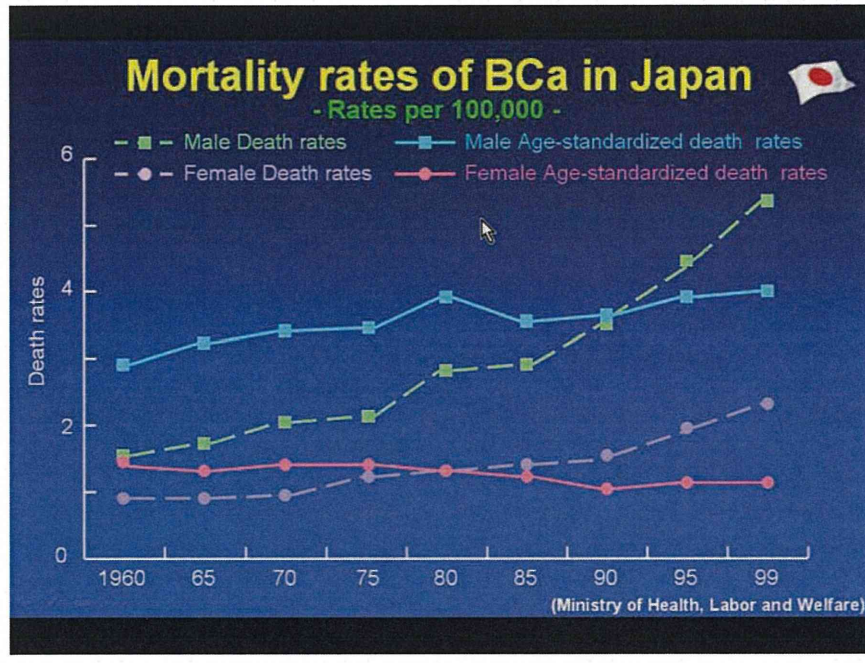

Figure 2. Mortality rates of bladder cancer in Japan.

case-controlled, population-based study in Michigan, USA (6). The results showed a higher risk of bladder cancer for those who worked for 20 or more years on the assembly line, with an odds ratio of 2.10 (95% CI: 1.15–3.80). Moreover, statistical interaction was shown between usual employment on the assembly line and smoking status, and the odds ratio was very high for smokers who had worked for a long time. It was concluded that further research is necessary to identify the exposures that might be contributing to bladder cancer on the assembly line and to examine whether those exposures continue to exist in today's workplace.

The environmental risks for bladder cancer include drinking water. Arsenic exposure is a well-known risk factor, and a very recent publication reported that chronic arsenic exposure at levels found in US drinking water was associated with bladder cancer (7). A population-based, case-controlled study conducted in New Hampshire in the USA consisted of

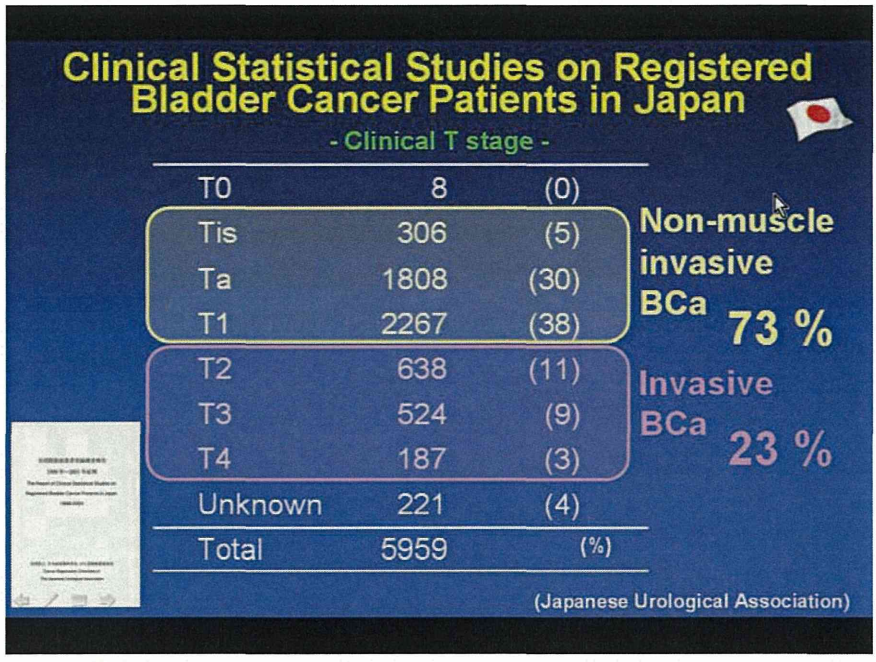

Figure 3. Clinical statistical studies on registered bladder cancer patients in Japan.

Table 2. Occupational high risk for blader cancer

Bitumen workers (asphalt, tar)
Automobile industries
Hair dressers exposed to colorants
Sewing machinests
Painters
Printers and paper hangers
Truck drivers
Etc.

832 cases of bladder cancer. Cases experienced a de-escalated survival hazard ratio versus a low (meaning less than the 25th percentile) toenail arsenic overall survival hazard ratio of 0.5. The population with a lower arsenic concentration was at lower risk. The bladder cancer cause-specific survival showed a similar trend, but did not reach statistical significance.

With regard to trihalomethanes (THMs), a 2007 report dealt with bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering and swimming in pools (8). More than 1200 bladder cancer cases and more than 1200 control subjects were included in a case-controlled study in Spain in 1998–2001. The results showed that long-term exposure to THMs was associated with a 2-fold bladder cancer risk, with an odds ratio of 2.10, for average household THM levels of >49 versus <8 μg/l. The odds ratio for showering/bathing was 1.83, whereas the odds ratio for swimming in a pool was 1.57 (Fig. 4).

The impact of dietary habits on the risk of bladder cancer was investigated by means of a principal components analysis case-controlled study conducted in Uruguay and reported in 2008 (9). The study subjects were 255 cases of newly diagnosed urothelial cancer and 501 hospitalized control subjects. In the case of a sweet beverage pattern characterized by high loading with coffee or tea with added sugar, the odds ratio was 3.27 and the 95% CI was 1.96–5.45. For a Western dietary pattern of high loading with red meat, fried eggs, potatoes and red wine, the odds ratio was significantly high at 2.35.

A 2008 study conducted in Canada investigated for associations of meat and fish consumption with various cancers (10). Nearly 20 000 patients with cancer of the stomach, colon, rectum, pancreas, lung, breast, prostate, bladder, etc., completed a questionnaire. The study included over 5000 population controls between 1994 and 1997 in eight Canadian provinces. The results indicated that total meat and processed meat consumption were directly related to the risk of various cancers: stomach, colon, rectum, pancreas, lung, breast, prostate, testis, kidney, bladder and leukemia. Also, red meat was significantly associated with colon, lung (mainly in men) and bladder cancer (Fig. 5). A second report, published in 2009, investigated meat intake and bladder cancer risk in a Swedish prospective cohort. In 1997, 82 002 Swedish women and men who were free of cancer completed a food-frequency questionnaire and were then prospectively observed (11). During a mean follow-up of 9.4 years, 485 cases of bladder cancer were diagnosed, but there was no association between the intake of total or any specific type of meat. This is thus a negative report as to the impact of meat intake.

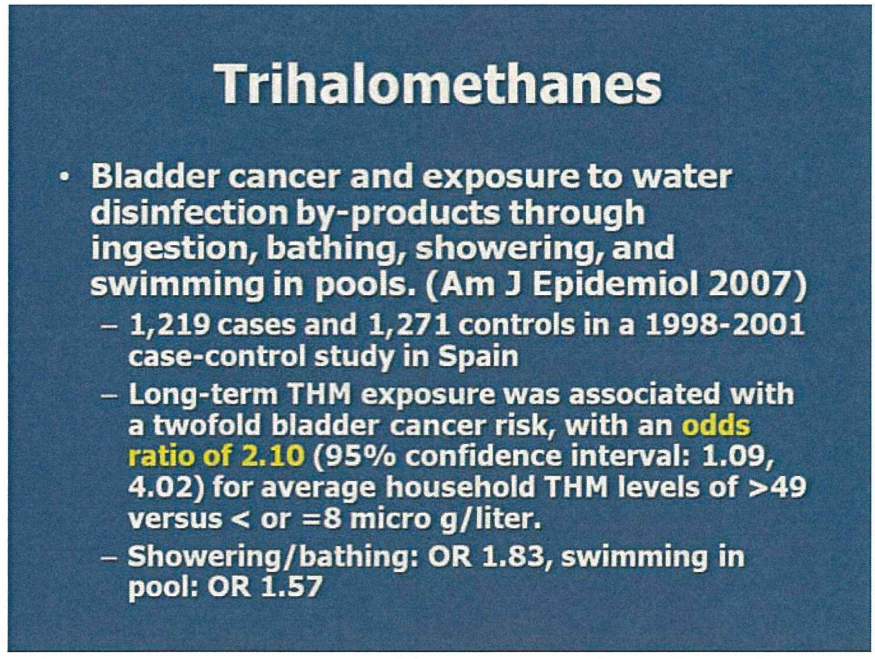

Figure 4. Trihalomethanes.

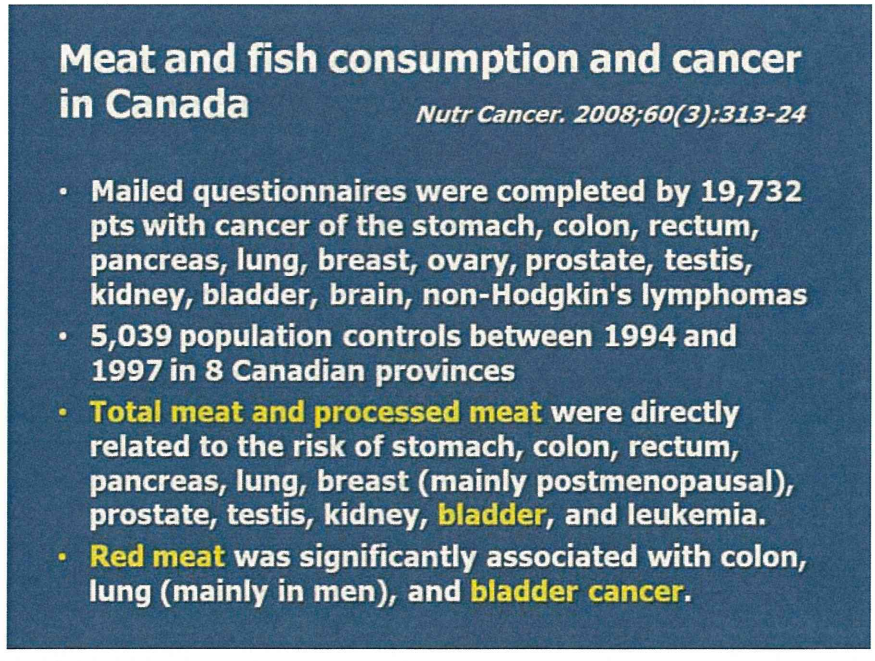

Figure 5. Meat and fish consumption and cancer in Canada.

Finally, with regard to cigarette smoking, it has been found that smoking accounts for more than 50% of bladder cancers in men and 30% in women. Patient awareness of smoking as a risk factor for bladder cancer was addressed in a 2009 published report (12). A prospective observational study investigated 202 consecutive urological inpatients by the use of a structured questionnaire (Fig. 6). Only 118 of the patients (58.4%) stated that they were aware of smoking as a risk factor for bladder cancer, which was strikingly lower than the 94.6% awareness with regard to chronic obstructive pulmonary disease, the 91.6% awareness with regard to heart and vascular problems and the 92.1% awareness with regard to lung cancer.

In conclusion, although the relative risk of bladder cancer associated with occupations is small, the public health impact may be significant. The Western pattern of diet was associated with a significant increase in the risk of bladder cancer. Urological patients' awareness of smoking as a risk

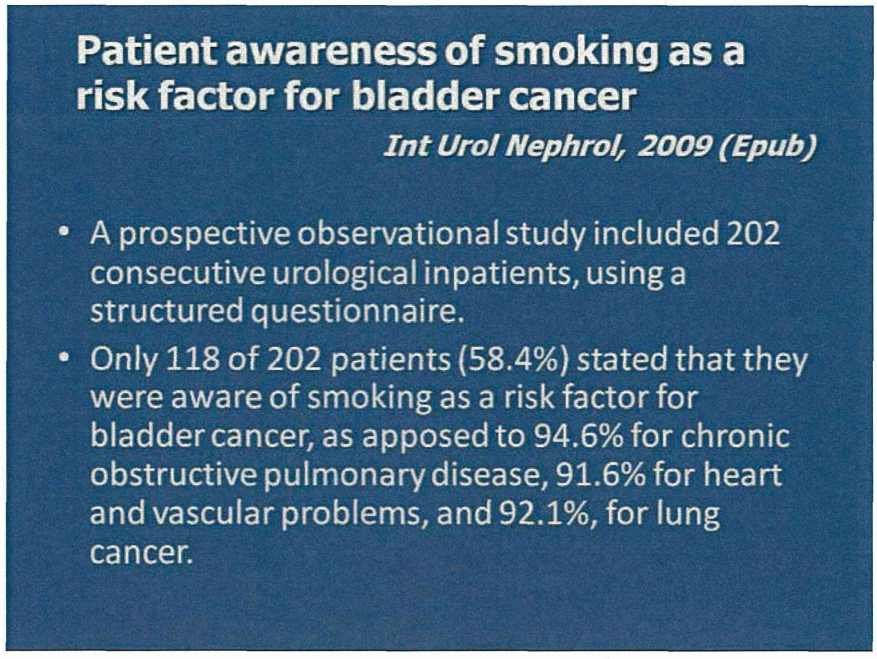

Figure 6. Patient awareness of smoking as a risk factor bladder cancer [Anastasiou et al. (12)].

factor for bladder cancer is lower than their awareness regarding other smoking-related disease entities. Counseling patients regarding the risk of tobacco is a role for urologists.

GENETIC SUSCEPTIBILITY TO UROTHELIAL CARCINOMA

Genetic variation in the human genome is an emerging resource for studying cancers, and it was reported that most population-attributable cancer heritability is related to polymorphic variation in the DNA sequence (13). An important point is that many of the genes encoding cytokines and enzymes are polymorphic.

N-acetyltransferase 2 is considered to be an important enzyme for detoxification of carcinogens, especially in bladder cancer. Slow and rapid acetylators have been shown to have variable association with bladder cancer (14–16). Racial differences have been shown for the prevalence of the slow acetylator genotype, with <20% in Asians and >55% in Caucasians (17,18). In Korean subjects, 7.5% of bladder cancer patients had the slow acetylator genotype (19). The lower incidence of this genotype in Asians may result in a lower incidence of bladder cancer.

A Korean study found that the risk of bladder cancer was increased in patients with tuberculosis and bronchial asthma (19). The reasons for this are unclear, but since patients with these chronic diseases undergo long-term drug treatment, perhaps the drugs or the disease processes are also influencing factors. On the other hand, the glutathione *S*-transferase T1 genotype is significantly associated with bladder cancer, and GSTM1 and GSTT1 appear to be important in detoxifying the products of oxidative stress (20,21). The frequencies of the GSTM1 and GSTT1 genotypes vary according to ethnicity (22,23). Western data suggest that null type GSTM1-negative and GSTT1-negative genotypes are significant risk factors for bladder cancer. In a Korean case-controlled study, a smoking history and a GSTT1 null genotype were significantly associated with bladder cancer risk, whereas in another population, the GSTM1 null genotype was not associated with bladder cancer risk (23). In uni- and multivariate analyses of environmental data from various countries and ethnic groups, a smoking history was possibly associated with bladder cancer, but the frequency of the GSTT1 null genotype was negatively associated with bladder cancer risk.

OGG1 8-oxoguanine DNA glycosylase is a DNA repair enzyme, and research has identified two polymorphic sites (24,25). One site was at codon 326 in exon 7, and the other was at codon 324 in exon 6. The association between bladder cancer risk and the codon 326 genotype in OGG1 was examined by age- and sex-adjusted analyses. The distribution of the codon 326 genotype in bladder cancer patients was significantly different from the control subjects (26). In particular, the bladder cancer risk in Korean males younger than 40 years old was approximately six times higher than in men older than 40 (27). Frequent mutation at codon 326 in OGG1 was found in bladder tumor tissues: ~24–25% of tumor tissues.

Tumor necrosis factor-α (TNF-α) is a very well-known cytokine. GA polymorphism at the −308 nucleotide of the TNF-α promoter was frequently seen in several cancers (28,29). Several studies reported that the cancer stage and grade were significantly associated with the GA genotype in the TNF-α promoter region (26,30). Examination of GA polymorphism in the TNF-α promoter region did not reveal

any difference between bladder cancer patients and control subjects. However, the relationship between high-grade bladder cancer and the −308 genotype, especially the GA genotype, was statistically significant. The GA genotype at this site had a statistically significant impact on TNF-α production and was also associated with a statistically significant increase in gene transcription. Moreover, the serum concentration of TNF-α was significantly higher in bladder cancer patients than in the control subjects.

Patients and controls were analyzed to determine whether the genetic polymorphisms in these five genes are risk factors for bladder cancer, and OGG1, GSTM1 and GSTT1 were found to be significantly associated with increased bladder cancer risk (26). Also, in uni- and multivariate analyses, the OGG1 and GSTMI genotypes were significantly associated with recurrence and progression, respectively, of non-bladder cancer. Collectively, data show that these single-nucleotide polymorphism (SNP) markers may be good indicators of the prognosis of bladder cancer in the clinical setting.

Recently, a Chinese group investigated the risk of bladder cancer in relation to the CYP, NAT2, GSTM1 and GSTT1 genotypes by stratifying the patients on the basis of their smoking history (31). The data showed that, in smokers, the NAT2 acetylation, GSTM1 null genotype and GSTM1/GSTT1 double null genotype were significant risk factors for bladder cancer. Subtype analysis showed that the NAT2, GSTM1 and GSTM1/GSTT1 null genotypes were associated with the tumor stage or grade. Another paper published in 2009 provided the best evidence that the GSTM1 null genotype and the NAT2 slow-acetylator genotype are associated with genetic susceptibility to bladder cancer (32).

In conclusion, recent SNP genetic studies in relation to bladder carcinogenesis have revealed several genetic polymorphisms of detoxification or DNA repair genes, such as NAT2, GST and OGG1. That information is important in relation to environmental risk factors and ethnic differences and will help predict the prognosis of patients with bladder cancer. Further studies are needed to confirm potential gene–gene and gene–environmental interactions leading to bladder carcinogenesis. Treatment of bladder cancer is completely dependent on the nature of the bladder cancer cells. If it is non-invasive, it can be treated by bladder preservation, using cystoscopic resection. On the other hand, if it is muscle-invasive, it may have high malignant potential for invasion or metastasis. Such patients should be treated by total cystectomy.

Conflict of interest statement

None declared.

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