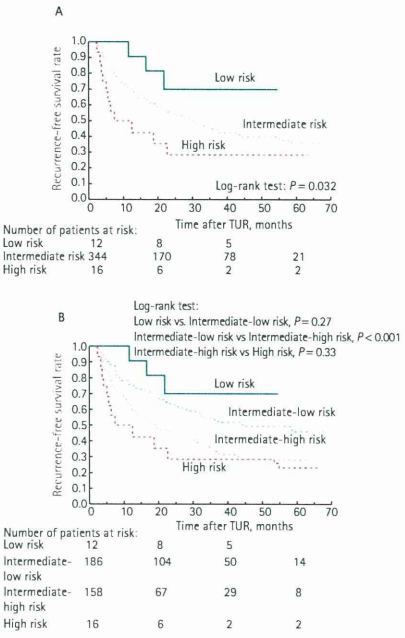
predicting recurrence after TUR in Japanese patients with stage Ta and T1 bladder tumours, and validated the EAU guidelines on risk group stratification to predict recurrence.

In the present study, ECOG PS, prior recurrence rate, number of tumours and T category were found to be independent predictors for time to recurrence in Japanese patients. Numerous publications have reported the prognostic indicators for tumour recurrence in bladder cancer not invading the muscle [3,11–14]. Kikuchi *et al.* [14] reported that the multiplicity of bladder tumours, tumour size >3 cm, pathological stage T1, tumour grade G3 and the absence of adjuvant intravesical instillation were independent risk factors for tumour recurrence in Japanese patients. There were some discrepancies concerning the predictive factors for recurrence between their study and the present study. In their study, only initially diagnosed bladder tumours not invading the muscle were evaluated, and tumours with concomitant CIS were excluded. However, as there are a number of patients with recurrent tumours not invading bladder muscle or with concomitant CIS, the prediction of recurrence could also be crucial for these patients, in terms of choosing the appropriate adjuvant treatment.

Interestingly, ECOG PS was independently associated with time to recurrence in multivariate analysis in the present study. PS has been reported to be an independent prognostic factor for patients with bladder cancer invading the muscle in several studies [15–18]. Matos *et al.* [15] indicated an independent prognostic value of ECOG PS for the disease-specific survival of patients with bladder cancer invading the muscle treated with TUR and chemotherapy, followed by radiotherapy or cystectomy. Yang *et al.* [16] showed that ECOG PS is one of the independent prognostic factors for survival of patients with bladder cancer invading the muscle treated with cystectomy or TUR with or without chemotherapy. Although, to date, there have been no reports on the association between PS and recurrence in patients with cancer not invading the bladder muscle, the results of the present study suggest that ECOG PS could potentially be a useful predictor for recurrence in these patients, as in patients with cancer invading bladder muscle. Possibly, patients with poor PS might have weakened immunological potency,

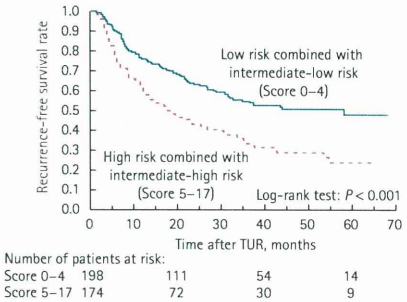
*FIG. 1. Kaplan–Meier recurrence-free survival curves for Japanese patients with Ta and T1 bladder tumours, stratified by the recurrence risk of EAU guidelines (P = 0.032, log-rank test) (**A**), and by the recurrence risk in which intermediate risk was further divided into intermediate-low risk and intermediate-high risk based on the EORTC risk table (P < 0.001 for intermediate-low risk vs intermediate-high risk, log-rank test) (**B**).*



which causes quicker bladder tumour recurrence.

In the present study of Japanese patients with Ta and T1 bladder tumours, most (92.5%) were classified into the intermediate-risk group according to the EAU guidelines for predicting recurrence [4], while only 3.2 and 4.3% patients were in the low- and high-risk groups, respectively. Several high-risk patients are likely to have been excluded due to the more radical treatments, including systemic chemotherapy, radiotherapy or cystectomy. The low frequency of low-risk patients could possibly depend on the lower rate of grade G1 tumours in the present study (17.7%) compared with the EORTC trials (43.2%) [3]. Because the rates of G1 tumours have been reported to be 24.2 and 22.4% in another Japanese study [14] and a Korean [13] study, respectively, there might be a racial difference in grade distribution of bladder tumours between Asian and Caucasian populations, similar to the difference between Caucasians and African-Americans [19]. The results of the

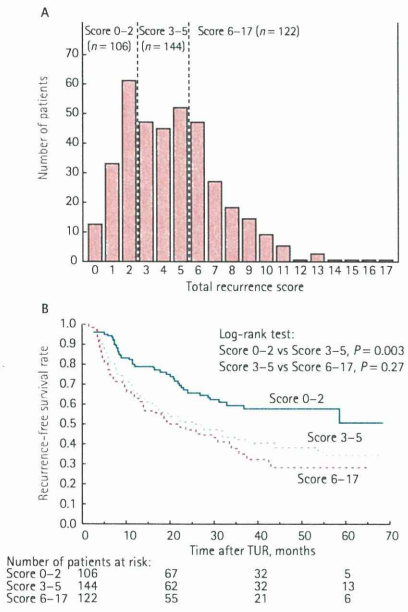
FIG. 2. Kaplan–Meier recurrence-free survival curves for Japanese patients with Ta and T1 bladder tumours, stratified by the two-tiered risk group for which the low risk and intermediate-low risk (score 0–4) groups were combined into one group, and the intermediate-high risk and high risk (score 5–17) groups were considered as another group (P < 0.001, log-rank test).



present study suggest that the risk group stratification of EAU guidelines for recurrence might not be a useful tool for Japanese patients with Ta and T1 tumours. Horvath and Mostafid [20] have stated that, whilst the therapeutic options for the low- and high-risk groups are well defined, the optimal treatment for patients in the intermediate-risk group is unknown. However, the intermediate-risk patients in the present study were further divided into intermediate-low-risk and intermediate-high-risk subgroups based on the EORTC risk table (total recurrence scores 1–4 and 5–9, respectively) [3], and a significant difference in the recurrence-free survival rates was found between these two subgroups. This result indicates that the subgroup classification of intermediate-risk patients might be appropriate to predict recurrence in Japanese patients, and could be essential for patient counselling, choosing the most appropriate adjuvant treatment after TUR, and determining the frequency of follow-up in individual patients.

In addition, it was also found that the patients with high risk combined with intermediate-high risk (total recurrence score 5–17) had significantly poorer recurrence-free survival rates than those with low risk combined with intermediate-low risk (score 0–4). This two-tiered risk group stratification could possibly simplify choosing the better adjuvant intravesical treatment for individual Japanese patients. In a meta-analysis of seven randomized trials, one immediate instillation of chemotherapy after TUR decreased the

*FIG. 3. Total recurrence score distribution for Japanese patients with Ta and T1 bladder tumours and three-tiered risk group stratification with nearly equal size to predict recurrence (score 0–2, n = 106; score 3–5, n = 144; score 6–17, n = 122) **(A)**, and Kaplan–Meier recurrence-free survival curves, stratified by this three-tiered risk group (P = 0.003 for score 0–2 vs score 3–5; P = 0.27 for score 3–5 vs score 6–17; log-rank test) **(B)**.*



percentage of patients with recurrence by 12% and the odds of recurrence by 39% [21], and the EAU guidelines recommend a single immediate instillation as adjuvant intravesical chemotherapy for patients with a low risk of recurrence [4]. Therefore, in Japanese patients with a score of 0–4, a single immediate instillation of mitomycin C, epirubicin or doxorubicin might be considered to be sufficient treatment. However, one meta-analysis comparing intravesical chemotherapy with TUR alone has shown that chemotherapy prevents recurrence but not progression [22], and another meta-analysis has shown that BCG therapy prevents, or at least delays, the risk of tumour progression [23]. Further chemotherapy or BCG immunotherapy might therefore be recommended for patients with a score of 5–17, and the choice of chemotherapy or BCG might depend on the progression risk of the individual patients. However, the data analysis using the two-tiered risk group stratification is exploratory, because this analysis was not performed after a pre-specified hypothesis or analysis plan. Thus, the usefulness of the two-tiered risk group stratification for recurrence in Japanese patients has to be confirmed in further studies.

In the EORTC risk table, to calculate the recurrence score, a weight (score) for each level of each variable was obtained based on the coefficients of the variables in the multivariate model, and the weights that corresponded to a given patient's characteristics were summed [3]. We reclassified the patients into three risk groups with nearly equal size based on the total recurrence score distribution (score 0–2, $n = 106$; score 3–5, $n = 144$; score 6–17, $n = 122$; Fig. 3A), and reanalysed the recurrence-free survival rates stratified by this three-tiered risk group (Fig. 3B). The patients with a score of 3–5 had significantly poorer recurrence-free survival rates than those with a score of 0–2 ($P = 0.003$, log-rank test); however, there was no significant difference between patients with a score of 3–5 and those with a score of 6–17 ($P = 0.27$, log-rank test). Thus, an altered weight (score) for each level of each variable calculated from data on a large number of Japanese patients is required for useful three-tiered risk group stratification with equal size to predict recurrence for Japanese patients.

In conclusion, ECOG PS, prior recurrence rate, number of tumours and T category were found to be independent predictors for time to recurrence after TUR in Japanese patients with stage Ta and T1 bladder tumours. In particular, ECOG PS could potentially be useful as a new predictor for recurrence. The present study is considered to be the first validation study of the EAU guidelines for recurrence of bladder tumour not invading the muscle. The risk group stratification of EAU guidelines for recurrence might not be applicable to Japanese patients, due to the numerical bias towards the intermediate-risk group. Instead of EAU risk stratification, two-tiered risk group stratification based on the EORTC risk table might be appropriate for Japanese patients with bladder cancer, to distinguish two different patient categories at risk of tumour recurrence. Further studies of a larger number of patients with a longer follow-up period, including data on tumour progression and disease survival, are required to help create original guidelines for Japanese patients with UC of the bladder that is not invading the muscle.

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CONFLICT OF INTEREST

None declared.

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Abbreviations: **CIS**, carcinoma *in situ*; **EAU**, European Association of Urology; **ECOG PS**, Eastern Cooperative Oncology Group performance status; **EORTC**, European Organization for Research and Treatment of Cancer; **HR**, hazard ratio; **TUR**, transurethral resection; **UC**, urothelial carcinoma.

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Could Patient Age Influence Tumor Recurrence Rate in Non-muscle-invasive Bladder Cancer Patients Treated with BCG Immunotherapy?

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Objective: The effect of local immunotherapy with bacille Calmette–Guérin in elderly patients with non-muscle-invasive bladder cancer has not yet been fully evaluated. The aim of the present study was to evaluate whether patients' age influences the response to bacille Calmette–Guérin treatment for the prevention of tumor recurrence and whether the side effects were tolerable.

Methods: We reviewed 1252 cases with non-muscle-invasive bladder cancer treated with transurethral bladder tumor resection, and 447 cases who underwent bacille Calmette–Guérin immunotherapy were included. The associations between patient age or pathological findings and tumor recurrence were determined. Side effects were classified as minor or major and were analyzed on the basis of their incidences in each age group.

Results: The patients were divided into four age categories: younger than 55 ($n = 86$), 55–64 ($n = 143$), 65–74 ($n = 132$) and equal or older than 75 years ($n = 86$). The Kaplan–Meier curves of recurrence-free survival rates demonstrated that patients aged 55–64 had been continuously tumor-free than the equal or older than 75 group. The presence of previous bladder cancer and Grade 3 were independent predictors for tumor recurrence; however, patients' age was not selected. The incidence of fever was slightly higher and that of cystitis was lower in the younger group.

Conclusions: Age does not certainly affect recurrence in patients with bladder cancer treated with bacille Calmette–Guérin therapy. The related side effects in the elderly patients were almost equal to those in the younger. With careful monitoring, bacille Calmette–Guérin therapy is safe even in elderly patients.

Key words: non-muscle-invasive bladder cancer – intravesical immunotherapy – age – side effect – bacille Calmette–Guérin

INTRODUCTION

Bladder cancer is a common disease in the elderly (1,2). Since the average life expectancy continues to increase, it is believed that bladder cancer will become an even more common disease in elderly people in the future. Most bladder tumors are non-muscle-invasive tumors (tumors confined to the mucosa and lamina propria). Non-muscle-invasive bladder tumors with high-risk factors are usually treated by transurethral bladder tumor resection (TUR-BT) and bacille Calmette–Guérin (BCG) immunotherapy. Of the patients who undergo complete TUR-BT for non-muscle-invasive bladder, 31–78% were reported to experience relapse within 5 years of follow-up (3). BCG has currently become the most commonly used intravesical agent and is known to be superior to other intravesical agents for the prevention of tumor recurrence (4).

BCG immunotherapy requires a robust immune system (5,6). Because elderly people have a comparatively weak immune system, they may not respond adequately to the treatment. On the other hand, serious severe side effects related to BCG immunotherapy cannot be ignored (7). The effectiveness and the side effect profile of BCG

immunotherapy should be re-evaluated, especially in elderly patients, and the upper age limit that would receive a benefit from BCG immunotherapy should be determined. The aim of the present study was to evaluate whether patients' age influences the response to BCG immunotherapy for the prevention of tumor recurrence and whether the side effects of BCG immunotherapy were tolerable by analyzing more than 400 Japanese cases with non-muscle-invasive bladder cancer.

PATIENTS AND METHODS

A review of the medical records of Keio University Hospital between 1981 and 2005 identified 1252 cases diagnosed with non-muscle-invasive bladder cancer (pTa or pT1) who were treated with TUR-BT. Among these cases, 447 (368 men and 79 women) underwent intravesical BCG immunotherapy after TUR-BT. Basically, we perform intravesical BCG therapy for intermediate- or high-risk non-muscle-invasive bladder cancers according to the current clinical guidelines. However, in some patients, whether or not intravesical therapy should be performed is mainly left up to the attending doctors' preference and the patient's wishes. We did not perform BCG immunotherapy in patients who had a past history of tuberculosis, and we confirmed that all patients receiving BCG immunotherapy did not have a strongly positive result in the tuberculin reaction. Their mean age was 64.7 years and the median follow-up period was 4.2 years (0.3–22.1 years). BCG immunotherapy was begun 4–5 weeks after TUR-BT and continued weekly for 6–8 weeks at a dose of 80 mg (Tokyo 172 strain). Patients were followed postoperatively with cystoscopy and urinary cytology every 3 months for 2 years, every 6 months for the next 3 years and then annually thereafter. Ultrasonography, excretory urograms and/or computed tomography were used to evaluate the bladder and upper urinary tract every year for 5 years after the treatment.

Side effects were classified as minor or major (8,9). BCG cystitis and hematuria symptoms that improved in <48 h with no subsequent recurrence were considered to be minor side effects, whereas those persisting for 48 h or longer were considered to be major side effects. BCG cystitis was defined as urinary frequency and/or pain in the hypogastrium. Low-grade fever subsiding within 2 days was considered to be a minor side effect, whereas fever persisting for 3 days or longer and/or a fever of 38°C or higher was considered to be a major side effect.

Tumor recurrence was defined as a new tumor appearing in the bladder. We determined the following patient characteristics: age, sex, the presence of previous bladder cancer, the number of tumors, tumor grade, pathological stage and the presence of carcinoma *in situ* (CIS), and analyzed the association between each parameter and tumor recurrence. The χ^2 test was used to analyze the difference between two groups. Univariate and multivariate analyses were performed using the Cox proportional hazard model to analyze the prognostic indicators for recurrence and to estimate the effect of patient age on the recurrence-free survival rate. The recurrence-free survival curves were constructed using the Kaplan–Meier method.

RESULTS

The patients were divided into four age categories for analysis: younger than 55 ($n = 86$), 55–64 ($n = 143$), 65–74 ($n = 132$) and equal or older than 75 years ($n = 86$). Table 1 shows the patient characteristics: sex, the presence of previous bladder cancer, the number of tumors, tumor grade, pathological stage and presence of CIS for each age category. Overall, Grade 3 (G3) tumors were found in 206 cases (46.1%), consisting of 107 cases with TaG3 tumors and 99 cases with T1G3 tumors. In the 154 cases (35.6%) with pathological stage T1 tumors, 55 cases had G1/2 tumors. Multiple tumors were seen in 251 cases (56.2%), and 54 cases (12.1%) had concomitant CIS lesions. There was no significant difference among the age categories. The mean ages of the patients with primary and recurrent tumors were 64.3 ± 10.8 and 66.0 ± 10.8 years, respectively ($P = 0.104$). The mean ages of the patients with G1/2 tumor and G3 tumor were 63.6 ± 10.4 and 66.1 ± 11.3 years, respectively ($P = 0.011$). The more elderly groups tended to include more patients with recurrent tumors and G3 tumors than the younger groups.

Figure 1 presents the recurrence-free survival rates after intravesical BCG immunotherapy according to the age category. The 5- and 10-year recurrence-free survival rates were 57.6 and 53.2% in the younger than 55 group, 63.3 and 47.6% in the 55–64 group, 58.5 and 47.9% in the 65–74 group and 50.6 and 35.8% in the equal or older than 75 group. Patients aged 55–64 had been continuously tumor-free than the equal or older than 75 group ($P = 0.029$); but among the four age categories, there was not another significant difference in the tumor recurrence rates. We performed univariate and multivariate analyses in order to define the predictors of tumor recurrence (Table 2). Univariate analysis showed that the presence of previous bladder cancer, G3 and pathological stage were prognostic indicators for tumor recurrence (Table 2). Multivariate analysis revealed that the presence of previous bladder cancer and G3 were independent predictors for tumor recurrence. However, the patient's age was not selected as an independent risk factor in either univariate or multivariate analysis.

Table 3 shows the side effects associated with BCG immunotherapy in each age category. Cut-off values of 55, 65 and 75 years of age were used to analyze in greater detail the differences in side effects. There was a statistically significant difference in BCG cystitis of side effect among the groups. The incidence of minor or major symptoms of cystitis in the younger than 55 group was 30.2%, which was slightly lower than that in the 55–64 group (48.3%), in the

Table 1. Patient characteristics according to the age group

	Total	Mean age ± SD (years)	<55 years old	55 ≤ age < 65	65 ≤ age < 75	≥75 years old	*P* value[a]
Sex							
Male	368	64.3 ± 10.5	74	119	110	65	0.2985
Female	79	66.7 ± 12.0	12	24	22	21	
Primary/recurrence							
Primary	325	64.3 ± 10.8	63	115	88	59	0.0587
Recurrence	122	66.0 ± 10.8	23	28	44	27	
Number of tumors							
Solitary	196	64.3 ± 10.3	37	67	57	35	0.8209
Multiple	251	65.1 ± 11.3	49	76	75	51	
Pathological							
Grade 1/2	241	63.6 ± 10.4	53	79	72	37	0.0984
Grade 3	206	66.1 ± 11.3	33	64	60	49	
Stage Ta	293	64.6 ± 10.4	61	97	82	53	0.4393
Stage T1	154	65.1 ± 11.6	25	46	50	33	
Concomitant CIS							
No	393	64.9 ± 10.5	74	127	118	74	0.8145
Yes	54	64.1 ± 13.1	12	16	14	12	

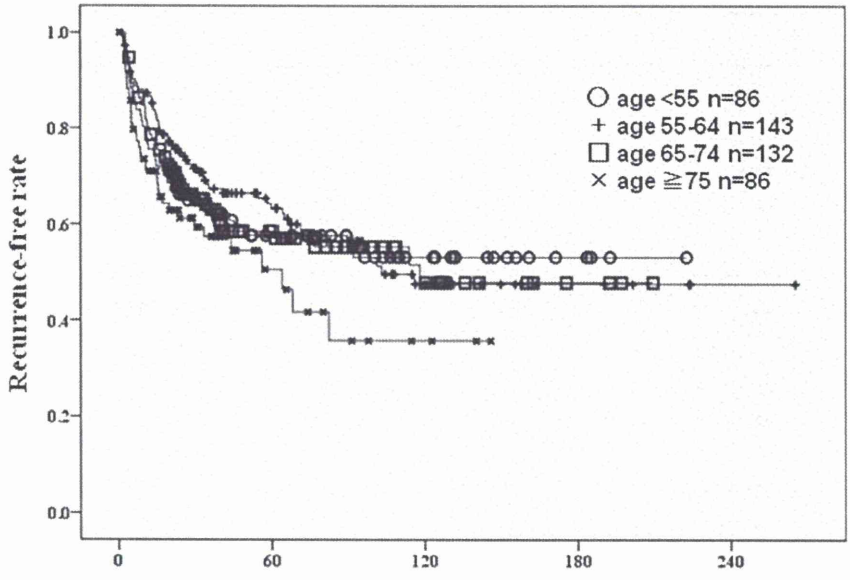
SD, standard deviation; CIS, carcinoma *in situ*.
[a]*P* value calculated by χ^2 test.

65–74 group (56.1%) and in the equal or older than 75 group (46.5%) ($P = 0.0074$, 0.0002 and 0.0282, respectively). On the contrary, the analyses showed that the incidence of fever was slightly higher in the younger group. Only 9.3% of patients in the equal or older than 75 group experienced major symptoms of fever, compared with 24.4% in the younger than 55 group, 18.9% in the 55–64 group and 18.2% in the 65–74 group ($P = 0.0081$, 0.0511 and 0.0702, respectively). Overall, only six cases (1.3%) were admitted due to a severe side effect, and there was no difference among the four groups.

DISCUSSION

The number of older people will continue to increase in the 21st century due to increases in median life expectancy. Some types of cancer, such as lung and stomach cancer, will occur more commonly in younger patients, whereas others such as uterine and ovarian cancer will be more common in elderly patients. Bladder cancer will also become more common in elderly people (10).

The immune system is thought to decline with age (11). Changes in T-lymphocytes and macrophages with age are well-accepted phenomena and result in reductions in phagocytic activity, cytokine [tumor necrosis factor-α, interleukin (IL)-2 and IL-6] and chemokine secretion, antibacterial defenses such as the production of reactive oxygen and nitrogen intermediates, wound repair function in the late phase of an inflammatory response and antigen presentation (12–14). These changes occur systemically in elderly people and lead to the impairment of immune defense mechanisms and a decreased capacity to contribute to the development of specific immune responses by presenting antigens to T cells and producing regulatory cytokines. Non-muscle-invasive bladder cancer treatment with BCG immunotherapy also relies heavily on the activation of cell-mediated immunity (15). Depressed immunity may be one of the causes for the low level of efficacy of BCG immunotherapy in elderly patients. However, it is still not totally clear as to whether elderly patients with non-muscle-invasive bladder cancer have a decreased response to BCG immunotherapy. Joudi et al. reported that patients more than 80 years old had a lower response to BCG plus interferon therapy than younger patients who were 61–70 years old. Multivariate analysis revealed that age was an independent risk factor for response ($P = 0.044$). They concluded that elderly patients with superficial bladder cancer have a poor response to intravesical immunotherapy. However, their immunotherapy was a combination of BCG and interferon (16). Herr reported that the 5-year recurrence-free survival rate in patients aged 70–79 was 26% compared with 40% in patients aged 50–69. In their study, multivariate analysis showed that being older than 70 years was an independent factor ($P = 0.03$) for cancer recurrence compared with younger patients (17). However, they also reported that 35% of the octogenarians



Number of patients at risk

age<55	86	34	16	4	0
age 55-64	143	60	20	6	1
age 65-74	132	41	13	3	0
age ≧ 75	86	12	3	0	0

Figure 1. Comparison of recurrence-free survival rate according to age.

Table 2. Results of univariate and multivariate analyses for tumor recurrence

	Univariate	Multivariate
Age	$P = 0.152$	
Sex (male vs. female)	$P = 0.390$	
Primary/recurrence (primary vs. recurrence)	$P = 0.005$	$P = 0.005$, HR: 1.572 (1.212–2.151)
Number of tumors (solitary vs. multiple)	$P = 0.305$	
Grade (G1/2 vs. G3)	$P = 0.045$	$P = 0.030$, HR: 1.377 (1.077–1.733)
Stage (pTa vs. pT1)	$P = 0.049$	
Concomitant CIS (yes vs. no)	$P = 0.556$	

responded to the treatment, which was about the same level as for patients aged 50–69. In our study too, the patients aged 55–64 had been continuously tumor-free than the equal or older than 75 years group but the younger than 55 group or the 65–74 group had the same level as that for the older than 75 years group, suggesting that tumor features other than advanced age seemed to determine the outcome. Mulders et al. found in a multivariate analysis that age had no association with the prognosis. However, the age cut-off point was 65 years (18). In the present study, there was no significant difference in tumor recurrence among the groups, and the multivariate analysis also demonstrated that the patient's age as a continuous value was not selected as an independent risk factor. Our results indicated that in patients treated with intravesical BCG immunotherapy, age was not correlated with the effect on reducing tumor recurrence. In this study, we divided the patients into four age categories. In some previous studies, the patients were stratified into five age categories for analysis with various cut-off levels (16,17). Dividing our patients into five age categories, we could construct recurrence-free survival curves. The curves demonstrated results similar to those of our study when the patients were divided into four age categories. However, in our study, the number of patients aged 80 or older was relatively small (only 27 patients), so we combined them into the 75 years and older group and then divided into four categories.

Yossepowitch et al. (19) evaluated the efficacy of BCG immunotherapy in steroid-treated and immunocompromised patients and reported a response rate at 6 months that was comparable to that in patients with no evidence of immunosuppression. Palou et al. (20) demonstrated that BCG immunotherapy could be safely administered to patients

Table 3. BCG-related side effects in the four age groups

	Total	<55	55 ≤ age < 65	65 ≤ age < 75	≥75
Admission for treatment of side effect	6 (1.3%)	1 (1.2%)	3 (2.1%)	1 (0.8%)	1 (1.3%)
Type of side effect					
Hematuria					
No	307 (68.7%)	61 (70.9%)	91 (63.6%)	88 (66.7%)	67 (77.9 %)
Minor	122 (27.3%)	21 (24.4%)	48 (33.6%)	38 (28.8%)	15 (17.4%)
Major	18 (4.0%)	4 (4.7%)	4 (2.8%)	6 (4.5%)	4 (4.7%)
Minor + major	140 (31.3%)	25 (29.1%)	52 (36.4%)	44 (33.3 %)	19 (22.1%)
Fever					
No	300 (67.1%)	50 (58.1%)	92 (64.3%)	94 (71.2 %)	64 (74.4%)
Minor	67 (15.0%)	15 (17.4%)	24 (16.8%)	14 (10.6 %)	14 (16.3%)
Major	80 (17.9%)	21 (24.4%)	27 (18.9%)	24 (18.2%)	8 (9.3%)
Minor + major	147 (32.9%)	36 (41.9%)	51 (35.7%)	38 (28.8%)	22 (25.6%)
BCG cystitis					
No	238 (53.2%)	60 (69.8%)	74 (51.7%)	58 (43.9%)	46 (53.5%)
Minor	170 (38.0%)	19 (22.1%)	59 (41.3%)	59 (44.7%)	33 (38.4%)
Major	39 (8.7%)	7 (8.1%)	10 (7.0%)	15 (11.4%)	7 (8.1%)
Minor + major	209 (46.8%)	26 (30.2 %)	69 (48.3%)	74 (56.1%)	40 (46.5%)
One symptom from above					
No	129 (28.9%)	28 (32.6%)	39 (27.3%)	31 (23.5%)	31 (36.0%)
Minor	196 (43.8%)	30 (34.9%)	64 (44.8%)	65 (49.2%)	37 (43.0%)
Major	122 (27.3%)	28 (32.6%)	40 (28.0%)	36 (27.3%)	18 (20.9%)
Minor + major	318 (71.1%)	58 (67.4%)	104 (72.7%)	101 (76.5%)	55 (64.0%)

BCG, bacille Calmette–Guérin.

with renal transplantation. These findings indicate that there is no clear association between the efficacy of locally administered BCG immunotherapy and the systemic immuno-response of the patient. The side effects associated with BCG immunotherapy are one of the factors that limit its safe continuation. The overall health condition of elderly patients is usually inferior to that of younger patients, which may put them at increased risk for systemic infections or the side effects of BCG. Therefore, it is generally assumed that BCG immunotherapy should be avoided in patients with immunosuppression. In our study, BCG cystitis, gross hematuria and fever, which we classified into major and minor side effects, were seen in 46.8, 31.3 and 32.9% of the patients, which were very similar to those reported in the previous studies (8,21). Although the incidence of fever was slightly higher and that of BCG cystitis was lower in the younger group (<55), the side effects of BCG immunotherapy in the elderly patients (≥75) were almost the same as those in the other age categories. In our study, the incidence of fever was slightly lower in the elderly group. There is general consensus that immunocompetence declines with age, and this might result in a low incidence of fever that is a systemic immune reaction to BCG. Meanwhile, BCG cystitis was more likely to be observed in the elderly group. The incidence of outlet obstruction related to BPH was relatively high in the elderly group. In this situation, BCG instillation could result in a strong reaction and effect in the bladder, which might have caused the increased BCG cystitis symptoms in the elderly patients. Heiner and Terris (22) reported an association between age and the side effects of BCG immunotherapy. In their study, the complication rates for patients <70 years old and those equal or older than 70 years on BCG immunotherapy were 17.6 and 48.6%, respectively, and the difference between the two groups was significant. However, their patients were treated with maintenance BCG immunotherapy. Moreover, the age cut-off point was 70 years in their study.

This study has several limitations. First, it was performed in a retrospective fashion. Second, no patients in our database underwent a second-look TUR-BT, immediate single instillation of chemotherapy after TUR-BT or any maintenance intravesical therapies, all of which may have improved the recurrence rates. Finally, no data concerning trouble with urination or medicines prescribed were incorporated into the analysis of side effects.

In conclusion, the age of patients with non-muscle-invasive bladder cancer treated with BCG immunotherapy did not seem to be associated with tumor recurrence. Moreover, the side effects of BCG immunotherapy in the elderly patients were almost the same as those in the younger patients. With careful monitoring, we believe that BCG immunotherapy can be safely administered to even elderly patients.

Conflict of interest statement

None declared.

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Copy number alterations in urothelial carcinomas: their clinicopathological significance and correlation with DNA methylation alterations

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The aim of this study was to clarify the genetic backgrounds underlying the clinicopathological characteristics of urothelial carcinomas (UCs). Array comparative genomic hybridization analysis using a 244K oligonucleotide array was performed on 49 samples of UC tissue. Losses of 2q33.3–q37.3, 4p15.2–q13.1 and 5q13.3–q35.3 and gains of 7p11.2–q11.23 and 20q13.12–q13.2 were correlated with higher histological grade, and gain of 7p21.2–p21.12 was correlated with deeper invasion. Losses of 6q14.1–q27 and 17p13.3–q11.1 and gains of 19q13.12–q13.2 and 20q13.12–q13.33 were correlated with lymph vessel involvement. Loss of 16p12.2–p12.1 and gain of 3q26.32–q29 were correlated with vascular involvement. Losses of 5q14.1–q23.1, 6q14.1–q27, 8p22–p21.3, 11q13.5–q14.1 and 15q11.2–q22.2 and gains of 7p11.2–q11.22 and 19q13.12–q13.2 were correlated with the development of aggressive non-papillary UCs. Losses of 1p32.2–p31.3, 10q11.23–q21.1 and 15q21.3 were correlated with tumor recurrence. Unsupervised hierarchical clustering analysis based on copy number alterations clustered UCs into three subclasses: copy number alterations associated with genome-wide DNA hypomethylation, regional DNA hypermethylation on C-type CpG islands and genome-wide DNA hypo- and hypermethylation were accumulated in clusters A, B_1 and B_2, respectively. Tumor-related genes that may encode therapeutic targets and/or indicators useful for the diagnosis and prognostication of UCs should be explored in the above regions. Both genetic and epigenetic events appear to accumulate during urothelial carcinogenesis, reflecting the clinicopathological diversity of UCs.

Introduction

Urothelial carcinomas (UCs) are classified as superficial papillary carcinomas or non-papillary carcinomas according to their configuration (1). Papillary carcinomas usually remain non-invasive although patients need to undergo repeated urethrocystoscopic resection for recurrences. In contrast, non-papillary invasive carcinomas usually develop from widely spreading flat carcinomas *in situ* showing a higher histological grade, and their clinical outcome is poor. There is also an alternative pathway by which papillary carcinomas develop higher histological grades during repetitive recurrence and transform into non-papillary invasive carcinomas. Thus, UCs show marked clinicopathological diversity (2). In order to improve the efficiency of diagnosis and therapy, it is necessary to clarify the genetic backgrounds underlying the various clinicopathological characteristics of UCs.

Previous studies employing Southern blotting based on restriction enzyme length polymorphism, polymerase chain reaction (PCR)–loss of heterozygosity (LOH) analysis using microsatellite markers, comparative genomic hybridization (CGH) analysis and fluorescence *in situ* hybridization (FISH) have revealed chromosomal instability in UCs such as losses of 2q, 5q, 9q and 10q and gains of 5p, 7p, 8q, 11q and 20q (3–12). However, such approaches are not effective for defining the break points in detail. Although recently developed array-based technology has been applied to UCs (13–18), the resolution of the arrays employed was insufficient or correlations between copy number alterations and the clinicopathological parameters of UCs were not analyzed in detail. Therefore, the genetic backgrounds underlying urothelial carcinogenesis have not been fully clarified.

In addition, multistage carcinogenesis is known to comprise both genetic and epigenetic events (19–21). We have reported the accumulation of DNA methylation on C-type CpG islands (22) in a cancer-specific, but not age-dependent, manner and demonstrated protein overexpression of DNA methyltransferase 1, a major DNA methyltransferase, even in non-cancerous urothelia with no apparent histological changes obtained from patients with UCs (23,24), as a result of possible exposure to carcinogens in the urine at the precancerous stage. Accumulation of DNA methylation on C-type CpG islands associated with DNA methyltransferase 1 protein overexpression was more frequently evident in aggressive non-papillary UCs (23,24). DNA hypomethylation on pericentromeric satellite regions was significantly correlated with LOH on chromosome 9 in UCs (25). Moreover, we have identified optimal indicators for carcinogenetic risk estimation in histologically normal urothelia, and for prognostication in surgically resected specimens from patients with UCs (26) using the bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) method (27–29), which is suitable for overviewing the DNA methylation tendency of individual large regions among all chromosomes (30). Although these data indicated that not only genetic but also epigenetic alterations play significant roles in UC development, to our knowledge, the correlations between copy number alterations and DNA methylation profiles in UCs have not been examined in a genome-wide manner.

In the present study of 49 UCs, we analyzed copy number alterations by array CGH analysis using a high-resolution (244K) oligonucleotide array, DNA methylation alterations on a genome-wide scale using BAMCA and DNA methylation status on C-type CpG islands using bisulfite modification. We then examined the clinicopathological significance of copy number alterations and the correlations between alterations of copy number and those of DNA methylation.

Abbreviations: BAC, bacterial artificial chromosome; BAMCA, bacterial artificial chromosome array-based methylated CpG island amplification; CGH, comparative genomic hybridization; COBRA, combined bisulfite restriction enzyme analysis; FISH, fluorescence *in situ* hybridization; LOH, loss of heterozygosity; mRNA, messenger RNA; MINT, methylated in tumor; MSP, methylation-specific polymerase chain reaction; RT, reverse transcription; PCR, polymerase chain reaction; UC, urothelial carcinoma.

Material and methods

Patients and tissue samples

Forty-nine samples (T1 to T49) of UCs of the urinary bladder, ureter and renal pelvis were obtained from specimens that had been surgically resected by radical cystectomy (16 patients) or nephroureterectomy (33 patients) at the National Cancer Center Hospital, Tokyo, Japan. The patients comprised 38 men and 11 women whose mean age was 68.59 ± 10.11 (mean ± standard deviation) years (range 49–85 years). Among the UCs, 19 and 30 were graded as low- and high-grade tumors, respectively, on the basis of the World Health Organization classification (31), and 34 and 15 were classed as superficial (pTis, pTa, pT1) and invasive (pT2 or more), respectively (31). Histological examination of UCs revealed lymph vessel involvement in 16 and vascular involvement in 9. On the basis of macroscopic examination, the UCs were divided into 28 papillary tumors and 21 non-papillary tumors. Five patients were positive for lymph node metastasis at the point of radical cystectomy or nephroureterectomy. Recurrence was diagnosed by urologists mainly on the basis of computed tomography, abdominal ultrasonography and urine cytological examinations. The mean observation period was 39.7 ± 31.8 months (mean ± standard deviation) and seven patients were positive for recurrence (lymph node metastasis, local recurrence and metastasis to the lung or bone in three, two and two patients, respectively). Clinicopathological parameters for each of the examined patients are summarized in supplementary Table S1, available at *Carcinogenesis* Online. This study was approved by the Ethics Committee of the National Cancer Center, Tokyo, Japan and was performed in accordance with the Declaration of Helsinki 1995. All patients gave their informed consent prior to their inclusion in this study.

Array CGH analysis

High-molecular-weight DNA from fresh-frozen tissue samples was extracted using phenol–chloroform, followed by dialysis. Array CGH was performed using a Human Genome CGH 244K Oligo Microarray Kit (Agilent Technologies, Santa Clara, CA). Labeling and hybridization were performed according to the manufacturer's protocol (Protocol v4.0, June 2006). Briefly, 2 μg of DNA from the patient and from a sex-matched control were double digested with AluI and RsaI (Promega, Madison, WI) for 2 h at 37°C. The digested DNA was then labeled by random priming using an Agilent Genomic DNA Labeling Kit Plus. Patient DNA and control DNA were labeled with Cy5-dUTP and Cy3-dUTP, respectively, and the labeled DNAs were hybridized with human Cot I DNA at 65°C with rotation for 40 h. Arrays were analyzed using the Agilent DNA microarray scanner and the Agilent Feature Extraction software. Presentation of the results was obtained using the Agilent CGH Analytics software package.

The results of array CGH were validated by FISH analysis. An LSI p16 (9p21) SpectrumOrange/CEP 9 SpectrumGreen Probe and an LSI p53 (17p13.1) SpectrumOrange Probe (Abbott/Vysis, Abbott Park, IL), corresponding to the CDKN2A and TP53 loci, respectively, were used. The FISH probes were hybridized to 5 μm thick sections of formalin-fixed, paraffin-embedded tissue specimens taken from a region immediately adjacent to that from which the corresponding fresh-frozen sample had been obtained within the same UC. Nuclei were stained with 4,5-diamidino-2-phenylindole.

BAC array-based methylated CpG island amplification

Because DNA methylation status is known to be organ specific (32), the reference DNA for analysis of the developmental stages of UCs should be obtained from the urothelium and not from other tissues or peripheral blood. Therefore, a mixture of normal urothelial DNA obtained from 11 male patients (C19 to C29) and 6 female patients (C30 to C35) without UCs was used as a reference for analyses of male and female test DNA samples, respectively. Of these 17 patients, 13 and 4 had undergone nephrectomy for renal cell carcinoma and nephrectomy for retroperitoneal sarcoma around the kidney, respectively. The mean age of the patients from whom normal urothelia had been obtained was 66.18 ± 10.49 (mean ± standard deviation) years (range 54–82 years). DNA methylation status was analyzed by BAMCA using a custom-made array (molecular cytogenetics Whole Genome Array-4500) harboring 4361 bacterial artificial chromosome (BAC) clones located throughout chromosomes 1–22, X and Y (33), as described previously (34,35). In 40 samples of UCs (T1 to T40), BAMCA had been performed and the results have already been published (26). For the present study, BAMCA was performed on nine additional samples of UCs (T41 to T49), and correlations between DNA methylation status and copy number alterations were examined in all 49 UCs.

Methylation-specific PCR and combined bisulfite restriction enzyme analysis

DNA methylation status on 5 C-type CpG islands was analyzed by methylation-specific polymerase chain reaction (MSP) and combined bisulfite restriction enzyme analysis (COBRA), as described previously (36). Briefly, bisulfite conversion was carried out using a CpGenome DNA Modification Kit (Chemicon International, Temecula, CA). DNA methylation status on CpG islands of the p16 and hMLH1 genes was determined by MSP using the primers described previously (36). The DNA methylation status of the methylated in tumor (MINT)-1, MINT-2 and MINT-12 clones was determined by COBRA using previously described primers and restriction enzymes (36). In 40 samples of UCs (T1 to T40), MSP and COBRA had been performed and the results have already been published (26). For the present study, MSP and COBRA were performed on nine additional samples of UCs (T41 to T49), and correlations between DNA methylation status and copy number alterations were examined in all 49 UCs.

Statistics

Correlations between copy number alterations and clinicopathological parameters of UCs were analyzed using the unpaired *T*-test. Based on Bonferroni correction for multiplicity of testing, differences at $P < 0.00714$ were considered significant. Unsupervised two-dimensional hierarchical clustering analysis of UCs was done using GeneSpring GX 10.0. Differences in the average number of array CGH probes showing copy number alterations, the average number of BAC clones showing DNA methylation alterations (hypo- and hypermethylation) and the average number of C-type CpG islands showing DNA methylation in UCs belonging to clusters A, B_1 and B_2 yielded by the unsupervised hierarchical clustering were analyzed using the Kruskal–Wallis test. Differences at $P < 0.05$ were considered significant.

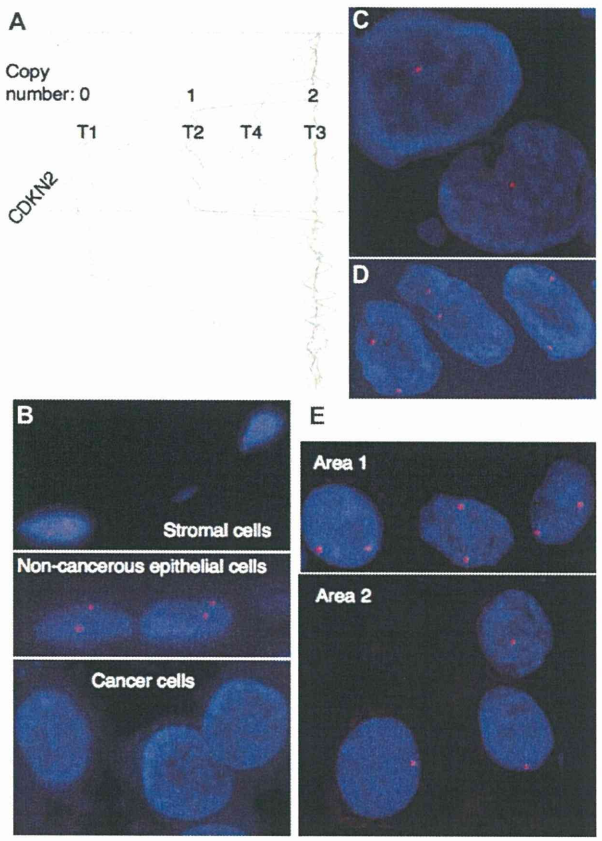

Fig. 1. Validation of array CGH analysis by FISH. (**A**) Array CGH profiles of representative tissue specimens (T1 to T4). The signal ratios of the CDKN2 locus in T1, T2 and T3 corresponded to copy numbers of 0, 1 and 2, respectively, whereas the signal ratio in T4 did not correspond to any whole number. (**B**) Although the LSI p16 (9p21) SpectrumOrange/CEP 9 SpectrumGreen Probe corresponding to the CDKN2A gene revealed two signals in stromal cells and adjacent non-cancerous urothelial cells, it revealed no signal in cancer cells in T1. (**C**) FISH analysis using the same probe revealed one signal in cancer cells in T2. (**D**) FISH analysis using the same probe revealed two signals in cancer cells in T3. (**E**) FISH analysis using the same probe revealed copy number heterogeneity in T4: cancer cells in areas 1 and 2 showed two signals and one signal within a tumor, respectively. These findings can explain the array CGH profile of T4 in panel (A).

Results

Validation of array CGH analysis by FISH

The array CGH analysis for copy number alterations was validated by FISH. Examples of array CGH profiles and FISH images of the four representative UCs (T1 to T4) are shown in Figure 1A–E, respectively. The signal ratios of the CDKN2A locus in T1, T2 and T3 corresponded to copy numbers of 0, 1 and 2, respectively, whereas the signal ratio in T4 did not correspond to any whole numbers (Figure 1A). The LSI p16 (9p21) SpectrumOrange/CEP 9 SpectrumGreen Probe corresponding to the CDKN2A gene revealed two signals in stromal cells and adjacent non-cancerous urothelial cells on the specimen of T1 (Figure 1B). The probe revealed zero, one and two signals in cancer cells in T1, T2 and T3, respectively (Figure 1B–D). FISH analysis revealed copy number heterogeneity within a UC: cancer cells showing two signals and those showing one signal were both observed in T4 (Figure 1E). These findings were able to explain the array CGH profile in T4 (Figure 1A). Similarly FISH analysis using the LSI p53 (17p13.1) SpectrumOrange Probe corresponding to the TP53 gene also validated the array CGH profiles (data not shown).

Copy number alterations and their clinicopathological impact in UCs

Figure 2 shows an overview of the copy number alterations on chromosomes 1–22 in all examined UCs. Chromosomal regions in which the incidence of copy number alterations in all examined UCs were ≥20% are summarized in Table I. If a UC showed copy number heterogeneity like that of T4 in Figure 1, the copy number observed in the major area within the tumor was described as the copy number of the UC in Figure 2 and Table I. On 3q26.1 and 4q13.2 (arrows in Figure 2), the incidence of homozygous deletion (copy number 0) on only 10 and 11 continuous oligonucleotide probes was high (59.2 and 67.3%, respectively, Table I). Although copy number polymorphism has been reported in the above region on 3q26.1, the UGT2B17 gene, which may be associated with smoking-related cancers (37), is the only gene reported to be located within the homozygously deleted region on 4q13.2.

Chromosomal loci on which copy number alterations were significantly correlated with clinicopathological parameters of UCs are shown in Figure 2. The clinicopathological impacts of the copy number alterations are also summarized in Figure 3. For example, loss of 1p32.2–p31.3 was correlated with UC recurrence. Loss of 2q33.3–q37.3 was correlated with higher histological grade. Gain of 3q26.32–q29 was correlated with vascular involvement. Loss of 4p15.2–q13.1 was correlated with higher histological grade. Losses of 5q13.3–q35.3 and 5q14.1–q23.1 were correlated with higher histological grade and tumor configuration (development of non-papillary tumors), respectively. Loss of 6q14.1–q27 was correlated with both lymph vessel involvement and tumor configuration. Gains of 7p21.2–p21.12, 7p11.2–q11.22 and 7p11.2–q11.23 were correlated with deeper invasion, tumor

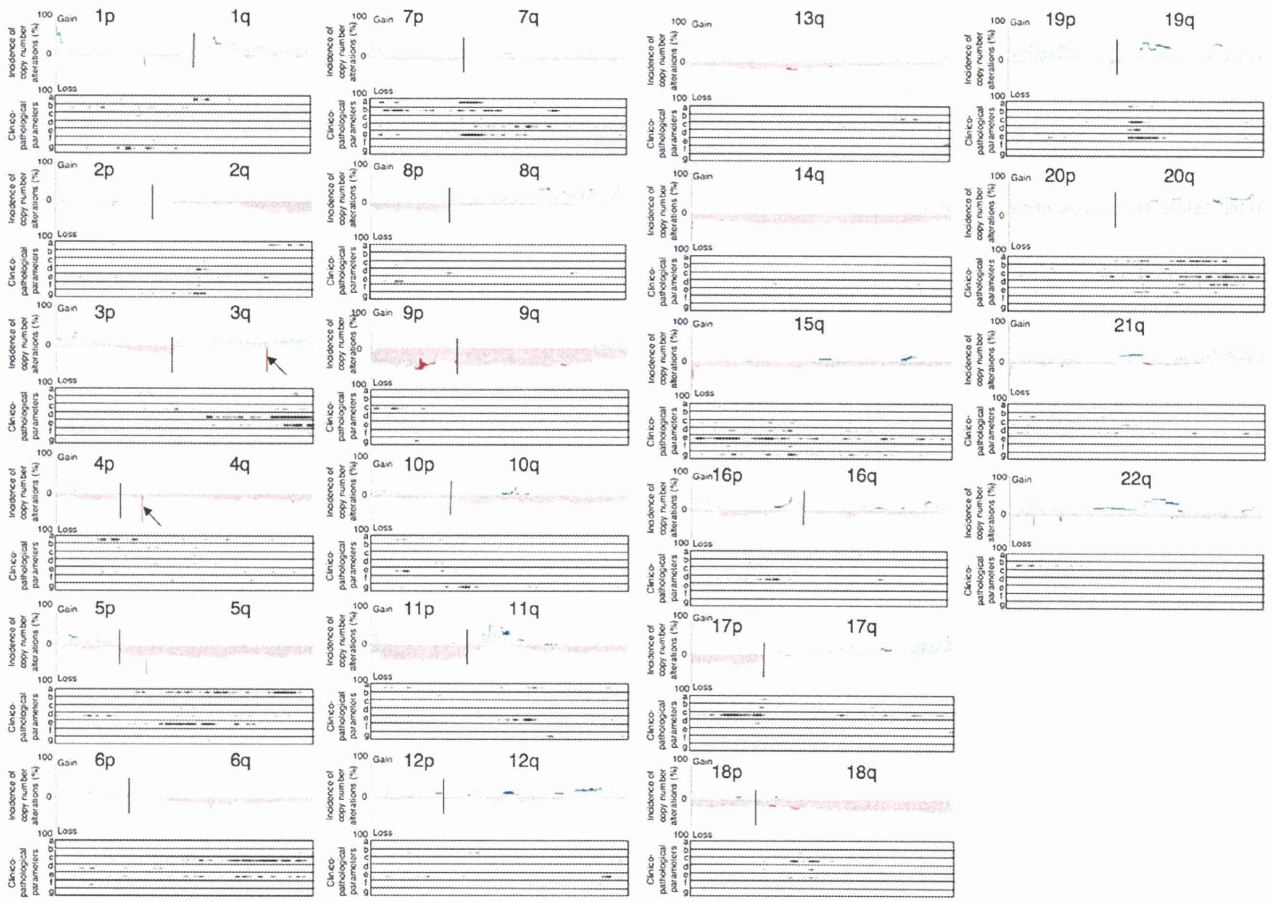

Fig. 2. Copy number alterations and their clinicopathological impacts in UCs. The incidence of copy number alterations on chromosomes 1–22 in UCs (T1 to T49) is shown. Gains (copy number: ≥3) and losses (copy number: 1 or 0) are shown in the upper and lower halves, respectively. Copy numbers of 0, 1, 3 and more are shown in dark red, light red, light blue and dark blue, respectively. The homozygously deleted regions on 3q26.1 and 4q13.2 are indicated by arrows. Locations of the array CGH probe on which copy number alterations were significantly correlated (unpaired *T*-test with Bonferroni correction, $P < 0.00714$) with histological grade (a), depth of invasion (b), lymph vessel involvement (c), vascular involvement (d), tumor configuration (papillary versus non-papillary, (e) lymph node metastasis (f) and recurrence (g) of UCs are indicated by 'X' under each of the histograms for chromosomes 1–22.

Table I. Copy number alterations showing incidences of >20% in the examined UCs

Chromosomal loci	Regions[a]	CN[b]	LI[c]	HI[d]	Chromosomal loci	Regions[a]	CN[b]	LI[c]	HI[d]
1p36.33–1p36.31	000554287–006656854	3	22.4	65.3	10q21.1–10q21.3	057989564–069900014	1	20.4	26.5
1p36.21–1p36.12	016167535–020948798	3	20.4	22.4	10q22.1–10q23.1	071904528–073262876	3	20.4	24.5
1p31.1	072550248–072568068	0	20.4	20.4	10q23.1	083089747–087102060	1	20.4	24.5
1q12–1q31.1	142721264–187457065	3	20.4	40.8	10q23.2–10q24.31	089445919–102120260	1	20.4	26.5
1q31.1–1q44	190299165–247190770	3	20.4	30.6	10q25.1–10q26.13	105709333–124981616	1	20.4	26.5
2q32.1–2q32.2	185846809–190742545	1	20.4	20.4	10q26.3	133783634–135066163	3	20.4	22.4
2q32.3–2q37.3	192350477–242717069	1	20.4	32.7	11p15.5–11p15.4	000182372–003270486	3	28.6	32.7
3p25.3	009517826–011146712	3	22.4	26.5	11q21–11q22.1	096386431–097377420	1	20.4	20.4
3p25.2–3p25.1	012038778–015468042	3	20.4	32.7	11q22.1–11q23.3	097429396–116543936	1	20.4	26.5
3p21.31	048346498–050680352	3	20.4	24.5	11q23.3–11q24.2	124714078–125280002	1	20.4	20.4
3p14.1	065390626–065748270	1	20.4	20.4	11q24.2	124714078–125280002	1	20.4	20.4
3p14.1–3p13	068561452–072819381	1	20.4	24.5	11q24.2–11q25	126760808–132830348	1	20.4	22.4
3p12.3	076229787–076504425	1	20.4	20.4	11q25	133367262–133662626	1	20.4	20.4
3p12.3	077466215–078924208	1	20.4	20.4	12p13.33	001767600–002412124	3	20.4	20.4
3p12.3	079019795–079019854	1	20.4	20.4	12p13.31	006172174–007239121	3	20.4	22.4
3p12.3–3p12.2	081674283–083271792	1	20.4	24.5	12q13.13–12q13.2	050511140–053289666	3	22.4	34.7
3q21.3	128095046–130849578	3	20.4	22.4	12q24.23–12q24.31	119012815–122594513	3	20.4	20.4
3q26.1	163997228–164101835	0	51.0	59.2	13q13.3	036216743–038434639	1	20.4	22.4
3q27.1–3q27.2	184336189–186044074	3	20.4	20.4	13q21.1	053204015–057297991	1	20.4	22.4
3q29	194947608–198141010	3	20.4	20.4	13q34	112711763–114123908	3	20.4	22.4
3q29	198287059–1993214468	3	20.4	20.4	14q11.1–14q11.2	018149473–019665348	1	20.4	40.8
4p16.3–4p16.2	000041413–004080171	3	22.4	30.6	14q12	025347676–028443093	1	20.4	20.4
4p16.2–4p16.1	004863024–009205888	3	20.4	30.6	14q12–14q32.31	028746840–101366386	1	20.4	36.7
4p16.1	009410429–009800836	3	20.4	20.4	14q32.33	103609874–105504791	3	20.4	30.6
4q13.2	069057735–069165872	0	38.8	67.3	15q11.1–15q11.2	018683110–020387386	1	24.5	36.7
4q28.3	135481517–138478960	1	22.4	26.5	15q11.2	018683110–019435559	3	20.4	20.4
4q34.1	172444145–173706467	1	20.4	20.4	15q21.3	054364049–055292702	1	20.4	20.4
4q34.2–4q35.1	176641828–183811059	1	20.4	24.5	15q24.1–15q24.2	072125930–073833248	3	20.4	20.4
4q35.1	184728685–184971082	1	20.4	20.4	16p13.3	000028087–003208434	3	22.4	36.7
4q35.1–4q35.2	186466884–190719413	1	20.4	24.5	16p13.2	007398132–007610763	1	20.4	20.4
5p15.33	000075149–004092634	3	20.4	46.9	16p11.2	028394123–031439837	3	20.4	32.7
5p15.32–5p15.2	005757937–010857368	3	20.4	22.4	16q21	057623929–059070656	1	20.4	20.4
5p15.2–5p15.1	014200030–016428467	3	20.4	20.4	16q22.1	065296755–066623461	3	20.4	22.4
5q11.1–5q35.3	049595677–180644869	1	22.4	63.3	16q24.1–16q24.3	083207007–088690615	3	20.4	26.5
6p21.33–6p21.32	031497746–032281493	3	20.4	24.5	17p13.3–17p11.2	000029169–020234630	1	20.4	32.7
6p21.32–6p21.31	033247001–034187994	3	20.4	20.4	17q11.2	023533773–024473421	3	20.4	20.4
6p21.2–6p21.1	040188750–044391792	3	20.4	24.5	17q21.2–17q21.31	037792629–038922402	3	20.4	20.4
6q16.3–6q21	101107740–105665855	1	20.4	22.4	17q21.31	039360337–041458716	3	20.4	20.4
6q21–6q22.2	113047208–117916793	1	20.4	20.4	17q21.32–17q21.33	043930335–046303881	3	20.4	24.5
7p22.2	000140213–003449208	3	20.4	42.9	17q24.3–17q25.3	067481954–078653589	3	20.4	49.0
7p22.2–7p22.1	003871971–005993219	3	20.4	28.6	18p11.32–18p11.23	000004316–008103527	1	20.4	22.4
7p13	043944978–045169498	3	20.4	24.4	18p11.21	013020518–013601674	1	20.4	20.4
7q11.23	072356188–075985576	3	22.4	22.4	18q11.2	019251951–019903282	1	20.4	20.4
7q22.1	099307676–102120122	3	20.4	30.6	18q12.1–18q23	023887204–076111023	1	20.4	36.7
8p23.2–8p23.1	002209252–006655643	1	20.4	22.4	19p13.3–19p13.11	000064418–019716580	3	28.6	53.1
8p23.1	007040596–008140129	1	24.5	32.7	19q12–19q13.42	032981858–061360576	3	20.4	42.9
8p22	013056908–018810539	1	20.4	22.4	20p13–20q13.33	000008747–062379118	3	26.5	57.1
8p21.3–8p21.2	023372368–027249779	1	20.4	24.5	21p11.2	009896630–013600286	1	20.4	34.7
8p21.1–8p12	027678573–037057454	1	20.4	28.6	21q22.3	041606431–046914745	3	20.4	38.8
8q11.1–8q24.3	047062121–146264902	3	20.4	61.2	22q11.21	016646613–019038934	3	22.4	44.9
9p24.3–9p11.2	000153131–044199460	1	20.4	53.1	22q11.21	018989547–018989606	0	20.4	20.4
9p11.2–9q34.3	045419207–140241935	1	20.4	51.0	22q11.21	019835358–020440240	3	20.4	24.5
9p21.3	021698371–022372349	0	20.4	26.5	22q11.23	021944430–022991816	3	20.4	22.4
9p12–9p11.2	041970428–046018111	3	26.5	36.7	22q12.3–22q13.1	034773534–038422701	3	20.4	40.8
9p24.3	000153131–140241935	1	20.4	53.1	22q13.3–22q13.33	049000786–049565875	3	20.4	20.4
9q34.2–9q34.2	135191259–139424835	3	20.4	22.4					

[a]Based on NCBI36/hg18.
[b]Copy number (If a UC shows copy number heterogeneity, the copy number observed in the major area within the tumor is considered to be the copy number of the UC).
[c]Lowest incidence of copy number alterations in the chromosomal regions (%).
[d]Highest incidence of copy number alterations in the chromosomal regions (%).

configuration and higher histological grade, respectively. Loss of 8p22–p21.3 was correlated with tumor configuration. Loss of 10q11.23–q21.1 was correlated with UC recurrence. Loss of 11q13.5–q14.1 was correlated with tumor configuration. Losses of 15q11.2–q22.2 and 15q21.3 were correlated with tumor configuration and recurrence, respectively. Loss of 16p12.2–p12.1 was correlated with vascular involvement of UCs. Loss of 17p13.3–q11.1 was correlated with lymph vessel involvement. Gain of 19q13.12–q13.2 was correlated with lymph vessel involvement and tumor configuration. Gains of 20q13.12–q13.2 and 20q13.12–q13.33 were correlated with higher histological grade and lymph vessel involvement, respectively. On the other hand, although the incidences of 8q gain and 9p, 11p and 14q loss were generally high in UCs, such copy number alterations were not evidently correlated with any clinicopathological parameters.

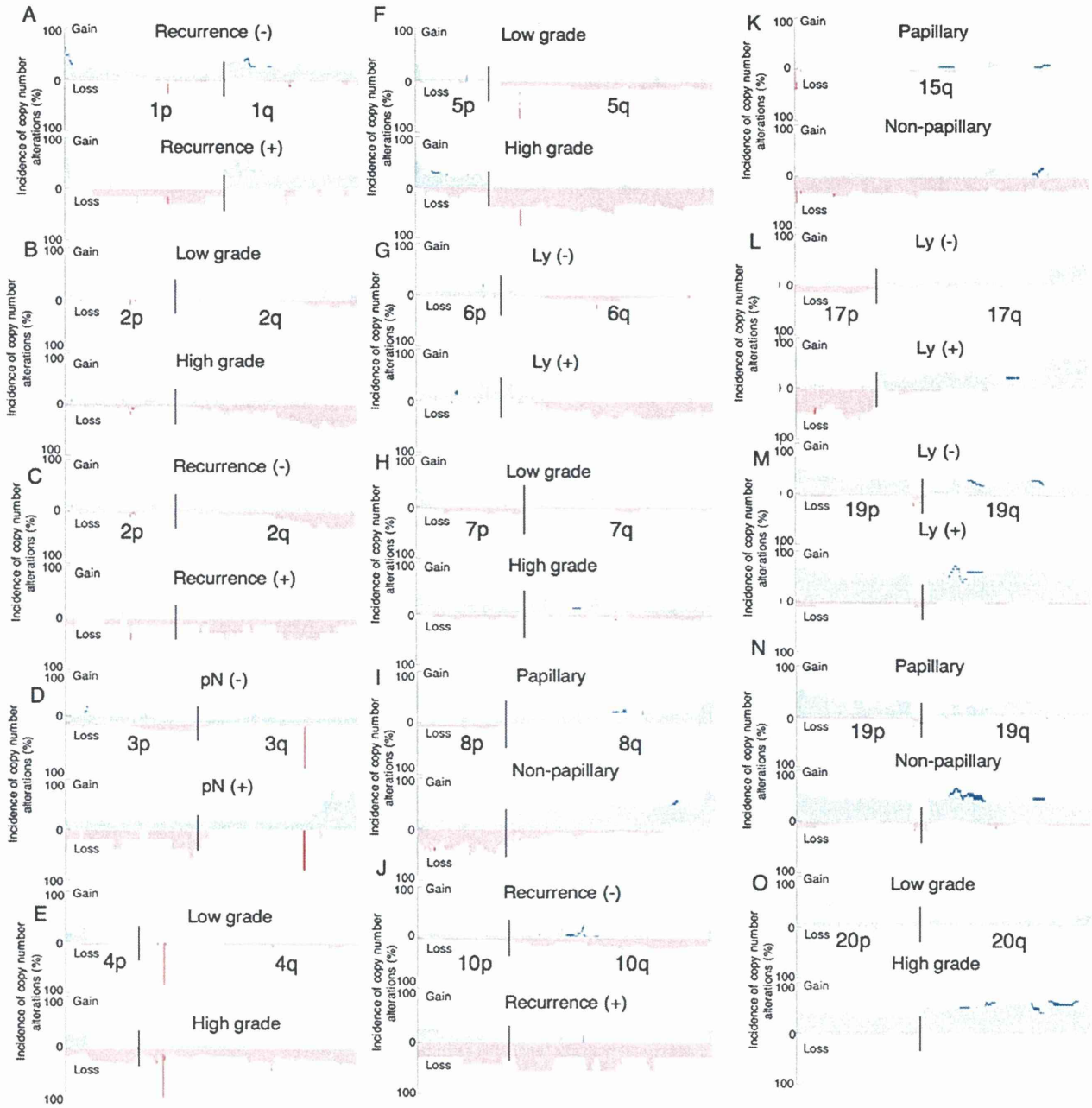

Fig. 3. Correlations between copy number alterations on representative chromosomes and clinicopathological parameters of UCs. The 49 UCs (T1 to T49) were divided into recurrence-negative (n = 42) and -positive (n = 7) cases (**A**, **C** and **J**), histologically low-grade (n = 19) and high-grade (n = 30) tumors (**B**, **E**, **F**, **H** and **O**), lymph node metastasis (pN)-negative (n = 44) and -positive (n = 5) tumors (**D**), lymph vessel involvement (Ly)-negative (n = 33) and -positive (n = 16) tumors (**G**, L and **M**), and papillary (n = 28) and non-papillary (n = 21) tumors (**I**, **K** and **N**). –, negative; +, positive. The incidence of copy number alterations on chromosomes 1 (A), 2 (B and C), 3 (D), 4 (E), 5 (F), 6 (G), 7 (H), 8 (I), 10 (J), 15 (K), 17 (L), 19 (M and N) and 20 (O) in each of the UC groups is shown. Gains (copy number: ≥3) and losses (copy number: 1 or 0) are indicated in the upper and lower halves, respectively. Copy numbers of 0, 1, 3 and more are shown in dark red, light red, light blue and dark blue, respectively.

Unsupervised hierarchical clustering of UCs based on array CGH data

Using two-dimensional unsupervised hierarchical clustering analysis based on copy numbers and all array CGH probes, the 49 UCs were clustered into three subclasses, clusters A, B_1 and B_2 (Figure 4), which contained 4, 12 and 33 tumors, respectively. The average number of probes on which loss (copy number 1 or 0) or gain (≥3) was detected was significantly higher in cluster A (99 499 ± 29 879) than in cluster B_1 (63 324 ± 40 064) and cluster B_2 (46853 ± 35000, P = 0.0271). As shown in Table II, the average number of probes on which gain (≥3) was detected was significantly higher in cluster A than in clusters B_1 and B_2 (P = 0.0153), whereas the difference in the average number of probes on which loss (1 or 0) was detected among clusters A, B_1 and B_2 did not reach statistical significance. The average number of probes on which a copy number of >3 was detected was significantly higher in cluster A than in clusters B_1 and B_2 (P = 0.0053). These data indicated

that copy number alterations, especially chromosomal gain, were accumulated in cluster A in comparison with clusters B_1 and B_2.

Correlation between genetic clustering of UCs and DNA methylation status revealed by BAMCA, MSP and COBRA

As shown in Table II, the average number of BAC clones showing DNA hypomethylation was significantly higher in cluster A than in clusters B_1 and B_2 ($P = 0.0487$), whereas there were no significant differences in the average number of BAC clones showing DNA hypermethylation among the three clusters. The incidence of DNA methylation on CpG islands of the p16 and hMLH1 genes and the MINT-1, MINT-2 and MINT-12 clones was 11 of 49 (detected/analyzed, 22.4%), 1 of 49 (2.0%), 9 of 49 (18.4%), 1 of 49 (2.0%) and 11 of 49 (22.4%), respectively. As shown in Table II, the average number of methylated C-type CpG islands was significantly higher in cluster B_1 than in clusters A and B_2 ($P = 0.0412$). Taken together, the data suggested that copy number alterations associated with overall DNA hypomethylation and regional DNA hypermethylation on C-type CpG islands were accumulated in clusters A and B_1, respectively, when defined on the basis of copy number alterations.

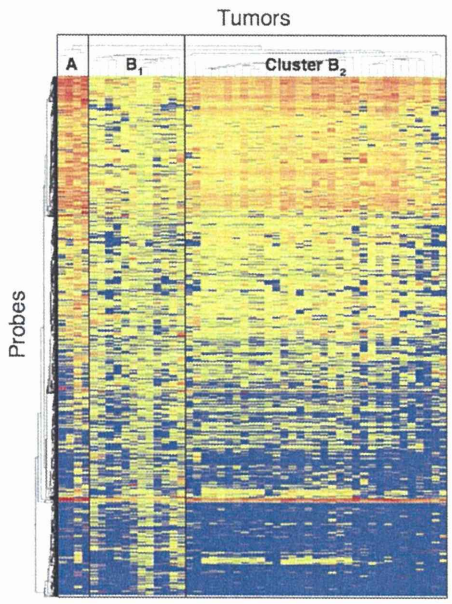

Fig. 4. Unsupervised two-dimensional hierarchical clustering analysis based on array CGH analysis of UCs (T1 to T49). Forty-nine patients with UCs were hierarchically clustered into three subclasses, clusters A ($n = 4$), B_1 ($n = 12$) and B_2 ($n = 33$), based on copy numbers. Copy numbers of 0 or 1 (loss), 2 (no change) and ≥3 (gain) on each probe are shown in blue, yellow and red, respectively. The cluster trees for tumors and probes are shown at the top and to the left of the panel, respectively.

Discussion

We and other groups have demonstrated copy number alterations in UCs for each chromosome or chromosome arm by Southern blotting, PCR–LOH and CGH analyses (3,4,6,7,9–11,25). Several array CGH analyses of UCs have also been performed using tiling BAC arrays (15,16,18). However, such analyses were unable to define the break points in detail. We here examined copy number alterations in UCs using a high-resolution (244K) oligonucleotide array capable of defining break points more precisely.

Copy numbers not corresponding to whole numbers were detected in the array CGH profiles of some UCs. In such cases, FISH analysis revealed copy number heterogeneity even within a single UC (e.g. cancer cells showing both two signals and one signal can be seen in T4 in Figure 1E). In UCs, heterogeneity of cellular atypia is frequently observed in histological specimens: a small area showing higher grade cellular atypia develops within a low-grade UC or cancer cells gain higher grade cellular atypia before they start to disrupt the basal membrane and invade into subepithelial tissues. It is feasible that copy number heterogeneity corresponds to such histological heterogeneity during the multistep malignant progression of UCs.

Our meticulous examination revealed the clinicopathological impacts of copy number alterations at various chromosomal loci (Figures 2 and 3). Losses (copy number 1 or 0) of 2q33.3–q37.3, 4p15.2–q13.1 and 5q13.3–q35.3 and gains (copy number ≥3) of 7p11.2–q11.23 and 20q13.12–q13.2 were significantly correlated with higher histological grade of UCs. Gain of 7p21.2–p21.12 was significantly correlated with deeper invasion. Losses of 6q14.1–q27 and 17p13.3–q11.1 and gains of 19q13.12–q13.2 and 20q13.12–q13.33 were significantly correlated with lymph vessel involvement. Loss of 16p12.2–p12.1 and gain of 3q26.32–q29 were significantly correlated with vascular involvement. Losses of 5q14.1–q23.1, 6q14.1–q27, 8p22–p21.3, 11q13.5–q14.1 and 15q11.2–q22.2 and gains of 7p11.2–q11.22 and 19q13.12–q13.2 were significantly correlated with tumor configuration (development of a non-papillary

Table II. Correlation between genetic clustering of UCs and copy number alterations and DNA methylation status

Copy number and DNA methylation status	Genetic clustering of UCs		
	Cluster A	Cluster B_1	Cluster B_2
Average numbers of array CGH probes showing copy number alterations			
Loss (1 or 0)	24 525 ± 15404	40 826 ± 31644	26 448± 28 462
Gain (≥3)	74 974± 38013	22 498 ± 15484	20 405 ± 17 369
Gain (>3)	1897 ± 1001[†]	236 ± 315	209 ± 361
Average numbers of BAC clones showing DNA methylation alterations			
DNA hypomethylation	312 ± 44[‡]	189 ± 95	236± 91
DNA hypermethylation	334± 85	254± 112	287± 77
Average numbers of C-type CpG islands showing DNA methylation	0.75 ± 0.96	1.33 ± 0.98[§]	0.58 ± 0.79

[*] $P = 0.0153$ to clusters B_1 and B_2.
[‡] $P = 0.0053$ to clusters B_1 and B_2.
[†] $P = 0.0487$ to clusters B_1 and B_2.
[§] $P = 0.0412$ to clusters A and B_2.

tumor). Possibly affected genes, which are located at such chromosomal loci and for which correlations with growth, motility and invasiveness of tumor cells and tumorigenesis have already been reported, are listed in supplementary Table S2, available at *Carcinogenesis* Online. Significant correlations between copy number alterations on such loci and clinicopathological parameters reflecting the malignant potential of UCs may be at least partly attributable to silencing or activation of the listed genes. Moreover, such chromosomal loci are important targets for exploration of unidentified tumor-related genes that participate in the malignant progression of UCs; the products of such genes may become target molecules for therapy of UCs. In addition, losses of 1p32.2–p31.3, 10q11.23–q21.1 and 15q21.3 were significantly correlated with recurrence of UCs: copy numbers at such chromosomal loci may become indicators for prognostication of patients with UCs (estimation of recurrence risk using surgically resected specimens).

On the other hand, although the incidence of gain of the entire arm of chromosome 8q and losses of the entire arm of chromosomes 9q, 11p and 14q were not significantly correlated with any of the examined clinicopathological parameters reflecting the malignant potential of UCs, the incidence of such copy number alterations was generally high. Such copy number alterations may occur in the earlier stage of development of both papillary and non-papillary UCs. Therefore, gatekeeper genes for urothelial carcinogenesis may exist on 8q, 9q, 11p and 14q. Combinations of the copy numbers of 8q, 9q, 11p and 14q could become applicable as indicators for the early diagnosis of UCs based on examination of urinary sediments and tissue specimens.

Moreover, the incidence of homozygous deletion on only 11 continuous oligonucleotide probes on 4q13.2 was high (67.3%) and the UGT2B17 gene is located within this homozygously deleted lesion. Copy number polymorphism of the UGT2B17 gene is reportedly associated with smoking-related cancer development (37), and a significant association between UCs and smoking has been demonstrated epidemiologically (38). Since there are many family genes, the exact copy numbers of the UGT2B17 gene were evaluated by quantitative PCR using specific primer sets (supplementary Table S3 is available at *Carcinogenesis* Online). Levels of expression of messenger RNA (mRNA) for the UGT2B17 gene normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA were also examined by quantitative reverse transcription (RT)–PCR analysis (supplementary Table S3 is available at *Carcinogenesis* Online) in 37 of the 49 UCs for which RNA samples were available. Quantitative RT–PCR data for the UGT2B17 gene in 28 UCs showing homozygous deletion (copy number 0) was 3.53 ± 6.40, being significantly lower than that in 9 UCs not showing it (61.61 ± 98.32, $P = 0.008176$). Since the homozygous deletion actually resulted in gene silencing, the correlation between the copy number of the UGT2B17 gene and susceptibility to UCs should be further examined.

UCs were grouped into three subclasses, clusters A, B_1 and B_2, based on copy number alterations. In cluster A, copy number alterations, especially chromosomal gains, revealed by array CGH analysis, and DNA hypomethylation revealed by BAMCA were both accumulated in a genome-wide manner. DNA hypomethylation may result in chromosomal instability through changes in chromatin configuration and enhancement of chromosomal recombination (39). Although such correlation between DNA hypomethylation and chromosomal instability has been observed in experimental models (40) and human immunodeficiency, centromeric instability and facial anomalies syndrome (41) and cancers (25,42), details of the DNA methylation status around each of the chromosome breakpoints are still unclear. UCs in cluster A may be ideal for examination of DNA methylation status around breakpoints to further clarify the molecular mechanisms responsible for chromosomal instability resulting from DNA methylation alterations. Cluster B_1 showed accumulation of regional DNA hypermethylation on C-type CpG islands. In addition, chromosomal losses tended to be accumulated in cluster B_1 in comparison with clusters A and B_2, although such differences did not reach statistical significance. The cancer phenotype associated with accumulation of DNA methylation on C-type CpG islands is defined as the CpG island methylator phenotype, and such accumulation is generally associated with frequent silencing of tumor-related genes due to DNA hypermethylation only and/or a two-hit mechanism involving DNA hypermethylation and LOH in human cancers of various organs (22). Silencing of tumor-related genes due to DNA hypermethylation and chromosomal losses may be critical for the development of UCs belonging to cluster B_1. In cluster B_2, the number of BAC clones showing both DNA hypo- and hypermethylation by BAMCA was rather high, and the number of probes showing loss or gain by array CGH was rather low, in comparison with cluster B_1, although such differences did not reach statistical significance. In addition to copy number alterations, genome-wide DNA methylation alterations may also participate in the development of UCs belonging to cluster B_2.

The number of CpG sites in CpG islands and repetitive sequences in 5′ regions, introns, exons and non-coding regions on BAC clones showing DNA hypomethylation in UCs are summarized in supplementary Table S4, available at *Carcinogenesis*. DNA hypomethylation was observed in BAC clones including both CpG islands and repetitive sequences, possibly resulting in activation of tumor-related genes and/or parasitic elements and loss of chromosomal integrity.

Silencing of representative genes on affected chromosomal loci was confirmed using quantitative RT–PCR analysis (supplementary Table S3 is available at *Carcinogenesis* Online). Although DNA methylation of the p16 gene was detected using MSP, quantitative examination using pyrosequencing (supplementary Table S3 is available at *Carcinogenesis* Online) revealed generally low DNA methylation levels (1.82 ± 0.65%) in all UCs. Therefore, correlations between copy numbers based on array CGH analysis and mRNA expression levels based on quantitative RT–PCR analysis were examined. The p16 gene was silenced in 11 UCs showing homozygous deletion (copy number, 0; quantitative RT–PCR data, 1.24 ± 1.20), whereas the mRNA expression level in 27 UCs not showing it was 104.1 ± 205.11 ($P = 0.00000357$). On the other hand, the DNA methylation level of the CXCL12 gene was 12.59 ± 18.43% for the UCs as a whole. The CXCL12 gene was silenced in 2 UCs with DNA methylation levels of ≥50% (mRNA expression level: 1.81 ± 1.00) but not in 34 UCs with DNA methylation levels of <50% (mRNA expression level: 24.45 ± 34.04). The level of expression of mRNA for the ERBB4 gene in 18 UCs showing a DNA methylation level of ≥5% and/or chromosomal loss (copy number 0 or 1) was 59.1 ± 101.2 and tended to be lower than that in 20 UCs with a DNA methylation level of <5% and a copy number of 2 (128.4 ± 259.3), suggesting the possibility of inactivation due to a combination of DNA hypermethylation and chromosomal loss, although such differences did not reach statistically significant levels. Taken together, the data suggest that genetic and epigenetic alterations (copy number alterations and DNA methylation alterations) are not mutually exclusive during urothelial carcinogenesis. Reflecting the clinicopathological diversity and histological heterogeneity of UCs, genetic and epigenetic events appear to accumulate in a complex manner during the developmental stage of individual tumors.

Supplementary material

Supplementary Tables S1–S4 can be found at http://carcin.oxfordjournals.org/

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Paclitaxel, ifosfamide, and nedaplatin as second-line treatment for patients with metastatic urothelial carcinoma: A phase II study of the SUOC group

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There is no standard second-line chemotherapy treatment for recurrent or metastatic urothelial cancer (MUC). The purpose of this phase II study was to evaluate the efficacy and toxicity of the three-drug combination of paclitaxel, ifosfamide, and nedaplatin (TIN). Patients with MUC were eligible after treatment failure with methotrexate, vinblastine, doxorubicin, and cisplatin, or gemcitabine and cisplatin. Doses for TIN therapy were paclitaxel 175 mg/m^2 on day 1, ifosfamide 1500 mg/m^2 on days 1–3, and nedaplatin 70 mg/m^2 on day 1, every 4 weeks. Tumor response, the primary efficacy parameter, was assessed according to unidimensional measurements (Response Evaluation Criteria in Solid Tumors criteria, version 1.0). Secondary efficacy parameters were overall survival (OS) and progression-free survival (PFS). Toxicity was assessed according to the National Cancer Institute Common Toxicity Criteria, version 3.0. A total of 45 patients (13 females and 32 males) with MUC were evaluable for response and toxicity. The overall response rate was 40.0%. Median PFS time was 4.0 months (95% confidence interval [CI], 4.6–11.6). Median OS time was 8.9 months (95% CI, 10.5–18.9). Grade 3 or 4 hematologic adverse events were neutropenia (95.6%), anemia (15.6%), and thrombocytopenia (17.8%). The most common grade 3 or 4 non-hematologic adverse events were anorexia (4.4%) and elevated aspartate transaminase/alanine transaminase (2.2%). No toxic death was observed. The main limitation of this study is that only 10 patients (22.2%) who were previously treated with gemcitabine and cisplatin were included. In conclusion, TIN as second-line treatment for MUC is an active regimen with a manageable toxicity profile. (*Cancer Sci* 2011; 102: 1171–1175)

Urothelial carcinoma of the bladder is the fourth most common cancer in men.[1] Systemic chemotherapy has been the mainstay of management for metastatic urothelial cancer (MUC).[2] Cisplatin-based combinations have evolved as the standard for first-line systemic therapy for MUC. The methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) regimen was reported to show an impressive complete remission rate of approximately 40–50% with substantial toxicity and a toxic death rate of 3–4% in MUC.[3,4] The gemcitabine and cisplatin (GC) regimen provides almost the same response rate as MVAC with less toxicity.[5] Neither of the two combinations proved superior over the other with prolongation of survival up to 14.8 and 13.8 months for MVAC and GC, respectively.[6] With cisplatin-containing combination chemotherapy, patients with lymph node metastases only, good performance status, and adequate renal function may achieve excellent response rates, including a high degree of complete responses, with up to 20% of patients achieving long-term disease-free survival.

However, no standard therapy has been established for patients pretreated for MUC.[7] Recently, new combination regimens, including paclitaxel and gemcitabine,[8–10] and paclitaxel and carboplatin,[11] indicated the efficacy and tolerability of paclitaxel-based therapy for the treatment of such patients. It has also been shown that ifosfamide is one of the most promising agents in salvage chemotherapy for MUC when it is given with paclitaxel[12] or gemcitabine.[13,14] Combination regimens using three agents, for example, paclitaxel, cisplatin, and methotrexate,[15] gemcitabine, ifosfamide, and cisplatin,[16] or paclitaxel, ifosfamide, and nedaplatin,[17] may provide higher response rates and longer survival than those with two agents. A preliminary study[18] indicated that nedaplatin had a higher inhibition index than cisplatin in all tissues from 12 urothelial cancer patients by the histoculture drug response assay.[19] The results suggest that nedaplatin can be effective for patients with progressive disease after cisplatin-based chemotherapies, such as MVAC or GC, although nedaplatin is cross-resistant to cisplatin.[20] Based on these data, this phase II study was carried out to evaluate the efficacy and toxicity of a regimen combining paclitaxel, ifosfamide, and nedaplatin (TIN) for MUC.

Materials and Methods

Eligibility. Patients eligible for study were those with a histological diagnosis of urothelial carcinoma of the bladder, ureter, or renal pelvis who had progressive or recurring disease, measurable disease according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria, were previously treated with MVAC or GC, aged ≥20 years, with an Eastern Cooperative Oncology Group performance status of 0–2 and with adequate bone marrow (neutrophil count ≥1000 cells/μL, platelets ≥50 000 cells/μL), hepatic (aspartate transaminase [AST] and alanine transaminase [ALT] ≤1.5 × upper limit of normal, unless there were liver metastases, in which case AST and ALT ≤5.0 × upper limit of normal), and renal function (creatinine clearance [CCr] ≥30 mL/min). Creatinine clearance was calculated according to the following formula: creatinine clearance (mL/min) = (urine creatinine/serum creatinine) × urine volume (mL)/(time [h] × 60]. All previous therapies for urothelial cancer had to be discontinued for ≥4 weeks before study entry and all acute toxic effects, excluding alopecia or peripheral neuropathy, of any prior therapy had to be recovered from. Life expectancy had to be ≥12 weeks and all patients needed to provide signed informed consent.

Exclusion criteria were: a serious uncontrolled medical disorder or active infection that would impair the ability to receive study treatment; known allergy or hypersensitivity to paclitaxel, ifosfamide, or nedaplatin; concomitant malignancy other than

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urothelial cancer; and female patients who were pregnant or lactating.

This study was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Approval to carry out the study was obtained from the institutional review board before the start of the trial.

Treatment plan. Paclitaxel was given at 175 mg/m^2 for 3 h by i.v. infusion on day 1 after premedication (given 1 h previous) consisting of dexamethasone 20 mg, ranitidine 50 mg, and diphenhydramine 50 mg. Ifosfamide was given at 1500 mg/m^2 per day i.v. on days 1, 2, and 3 for 4 h/day (total dose 4500 mg/m^2). Mesna uroprotection at 300 mg/m^2 i.v. was given with the ifosfamide infusion, then at 3 and 6 h afterwards. Nedaplatin was given at 70 mg/m^2 i.v., for 3 h on day 1 with vigorous pre- and post-hydration and mannitol. The chemotherapy schedule was recycled every 28 days. If the CCr was <60 mL/min, the doses of ifosfamide and nedaplatin were reduced to 3600 mg/m^2 and 70 mg/m^2, respectively, in the case of 45 ≤ CCr < 60 mL/min, and 3375 mg/m^2 and 35 mg/m^2, respectively, in the case of 30 ≤ CCr < 45 mL/min. If a complete response (CR), partial response (PR), or stable disease (SD) response was obtained and the disease site was considered to be resectable after TIN treatment, salvage surgery or radiation therapy was carried out.

Evaluation. Tumor response, the primary efficacy parameter, was assessed every two cycles according to RECIST criteria, version 1.0.[21] Complete response required the total disappearance of all evidence of cancer for at least 4 weeks. Partial response (PR) required a more than 30% reduction in the sum of the longest diameters of the target lesions without any new lesions for at least 4 weeks. The response status was reviewed by a panel of independent experts. Secondary efficacy parameters were overall survival (OS) and progression-free survival (PFS). The qualitative and quantitative toxic effects were graded in agreement with National Cancer Institute Common Toxicity Criteria, version 3.0.

Statistical analysis. For the primary end-point, the percentage of change in the sum of the longest diameter of the target lesion from baseline to the best response was calculated and recorded in a waterfall plot. For the secondary end-point, PFS was calculated from the first day of treatment to the date of the first documented disease progression or death from any cause. The Standard Southwest Oncology Group Phase II design[22] was used to plan this study on the assumption that the regimen would not be of interest if the true response rate was <20%, but that it would be of interest if the response rate was 40% or more. Alpha (the significance level) and 1-beta (the power) were set as 0.05 and 0.90, respectively. If more than four of the first 25 patients obtained a response, planned accrual of 20 more eligible patients was to be carried out. Overall survival was defined as the time between the first day of treatment and date of death from any cause. Patients remaining on-study or alive at time of analyses were censored at the date of the last follow-up. The probability of PFS and OS was estimated using the Kaplan–Meier method.

Results

Patient characteristics. Between April 2005 and January 2010, 45 patients with advanced transitional cell cancer were entered into this phase II study. Because a response was obtained in 36.0% of the first 25 patients, 20 additional patients were enrolled in this study. All patients were assessable for efficacy and safety. The baseline characteristics of the patients are presented in Table 1.

Extent of exposure. Altogether 108 cycles were given with a median of two cycles per patient (range, 1–6). In eight (17.8%) patients the drug doses were reduced due to renal dysfunction.

Table 1. Baseline characteristics of patients with metastatic urothelial carcinoma who participated in this study

Characteristic	*n*	%
No. of patients	45	—
Median age, years (range)	68 (35–78)	—
Male:female	32:13	—
ECOG performance status		
0	35	77.8
1	6	13.3
2	4	8.9
Primary site		
Bladder	26	57.7
Renal pelvis	13	28.9
Ureter	6	13.3
Metastatic sites		
Lymph nodes	31	68.9
Pelvic mass	5	11.1
Lung	15	33.3
Liver	9	20.0
Bone	6	13.3
Adrenal	1	2.2
Ovary	1	2.2
Duodenum	1	2.2
Peritoneum	1	2.2
Contralateral renal pelvis	1	2.2
No. of sites with tumor involvement		
1	25	55.6
2	14	31.1
≥3	6	13.3
Prior chemotherapy		
MVAC	35	77.8
GC	10	22.2

—, Not applicable; ECOG, Eastern Cooperative Oncology Group; GC, gemcitabine and cisplatin; MVAC, methotrexate, vinblastine, doxorubicin, and cisplatin.

Seven patients stopped therapy after only one cycle, either because of progressive disease (two patients), patient choice to withdraw from treatment (four patients), or the adverse effect of AST/ALT elevation (one patient). Fifteen (33.3%) patients were treated with three or more cycles of therapy.

Responses to treatment. Of the 45 patients, two CR and 16 PR were obtained (Table 2). Plots of serial tumor measurements over time and a waterfall plot of the best response, to better characterize antitumor activity, showed that 28 (62.2%) of the 45 patients had some degree of tumor reduction (Fig. 1). The overall response rate was 40.0%. Overall, at follow-up, five (11.1%) patients were progression free. The median PFS and OS were 4.0 months (95% confidence interval [CI], 4.6–11.6) and 8.9 months (95% CI, 10.5–18.9), respectively (Fig. 2). Responses stratified by the first-line chemotherapy and disease

Table 2. Best overall response to paclitaxel, ifosfamide, and nedaplatin as second-line chemotherapy regimen in 45 patients with metastatic urothelial carcinoma

Response	No. of patients (%)
CR	2 (4.4)
PR	16 (35.6)
SD	15 (33.3)
PD	12 (26.7)
Total	45 (100)

CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

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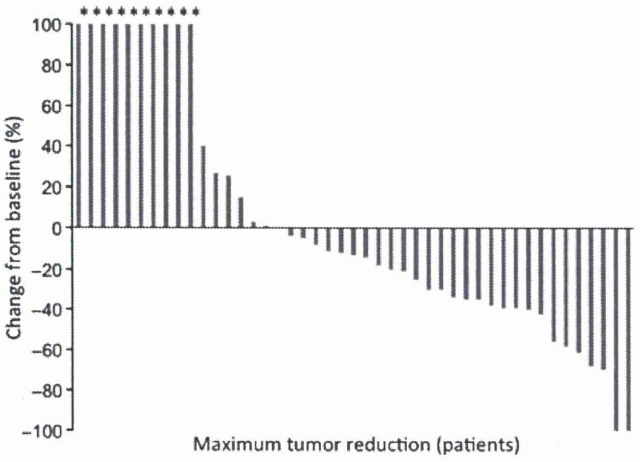

Fig. 1. Waterfall plot of percentage changes showing the best response in each patient with metastatic urothelial carcinoma who underwent second-line chemotherapy treatment with paclitaxel, ifosfamide, and nedaplatin. *Appearance of a new lesion.

site are shown in Tables 3 and 4, respectively. There was no significant difference in the objective response (OR) rate, PFS, or OS between patients who received MVAC and those who received GC as the first-line therapy (37.1% *vs* 50.0%, median 4.0 *vs* 5.6 months, and median 8.8 *vs* 9.9 months, respectively). Six (13.3%) patients (one CR, four PR and one SD) underwent salvage surgery or radiation therapy after TIN therapy. Of these six patients, three (50.0%) were followed-up for 50, 50, and 49 months after the salvage surgery without recurrence.

Toxicity. There were no toxicity-related deaths with TIN. Grade 3/4 leukopenia, neutropenia, thrombocytopenia, anemia, anorexia, and elevated AST/ALT were noted in 42 (93.3%), 43 (95.6%), 8 (17.8%), 8 (17.8%), 2 (4.4%), and 1 (2.2%) patients, respectively (Table 5). The median onsets of neutropenia and thrombocytopenia for all grades were both 11 days. Ten (22.2%) patients displayed febrile neutropenia and were treated with antibiotics until the neutrophil count recovered and the fever abated. Granulocyte-colony stimulating factor was given as a treatment for neutropenia a median of five times per course (range, 0–11). Peripheral neuropathy was observed in seven (15.6%) patients, but no grade 3/4 neuropathy occurred. Another common side-effect was alopecia in 100%.

Discussion

Although urothelial cancer is chemosensitive, there are insufficient data to provide a recommendation on standard second-line chemotherapy.[6] Various regimens have been investigated in an attempt to prolong survival for first-line chemotherapy-resistant MUC. In second-line chemotherapy for MUC, a paclitaxel-based regimen, such as paclitaxel and cisplatin, or paclitaxel and carboplatin, provided 30–70% OR rates and a median OS of 7.9–11.5 months.[8–11] Phase II trials of a gemcitabine/ifosfamide regimen had a 21–22% OR with a median OS of 4.8–9.0 months.[13,14] A paclitaxel/ifosfamide regimen gave a 15% OR with a median OS of 8 months.[12] Thus, combinations of two anticancer drugs had similar results, that is, a median OS of 6–12 months.

A phase II study of gemcitabine, ifosfamide, and cisplatin (gemcitabine 800 mg/m^2, ifosfamide 1000 mg/m^2, and cisplatin 30 mg/m^2, on days 1, 8, and 15 on a 28-day cycle) for MUC[16] provided an OR of 40.8% with a median OS of 9.5 months. However, the intended schedule of weekly doses given three times per cycle was not deliverable due to hematologic toxicity. Thus significant toxicity can be expected and is of concern, especially in patients who have undergone several courses of first-line chemotherapy. Shinohara *et al.*[17] invented the TIN regimen by modification of paclitaxel/ifosfamide/cisplatin[23] with the aim of higher relative dose intensity with lower toxicities for MUC patients who had received prior chemotherapy. They used nedaplatin, a second-generation platinum complex with lower renal and gastrointestinal toxicities than cisplatin,[24] instead of cisplatin, and reported a surprising result, a 75% OR (16% CR + 59% PR) with a median OS of 22 months.

The OR of 40% and the median survival of 8.9 months in this study are similar to those for paclitaxel/gemcitabine[8–10] rather than the first report on TIN by Shinohara *et al.*[17] One of the reasons explaining this is a difference in patient characteristics between the two studies of TIN. First, this study included only eight (17.8%) patients who had a CR or PR after the first-line chemotherapy, which indicated a good response to TIN (Table 3). In contrast, 14 (43.8%) such patients were treated by TIN in Shinohara's study. Another possibility is that Shinohara's study included fewer patients with visceral metastases (lung and liver metastasis in 18.8% and 6.3% of the patients, respectively) than others,[8–11] including this study. Nineteen (42.2%) of the patients in this study had multiple organ diseases, which showed lower responses to TIN than lymph node or lung metastasis (Table 4).

Grade 3/4 neutropenia, thrombocytopenia, and anemia were noted in 43 (95.6%), eight (17.8%), and seven (15.6%) patients, respectively. Although most of the patients displayed hematological toxicity, these adverse effects were manageable and no delay in the start of chemotherapy was required. One patient stopped the therapy because of AST/ALT elevation due to allergic hepatitis induced by the polyoxyethylated castor oil solvent used for paclitaxel. No other patient discontinued this treatment due to toxic effects. Thus, the TIN regimen was well tolerated with hematological toxicity being the most significant side-effect.

Salvage surgery after the chemotherapy might have favorably impacted the observed survival in this series. However, post-chemotherapy metastasectomy is still controversial. Dodd *et al.*[25] reported that salvage surgery after MVAC contributed to long-term survival with a 5-year survival of 33%. Abe *et al.*[26] showed the impact of metastasectomy on survival in

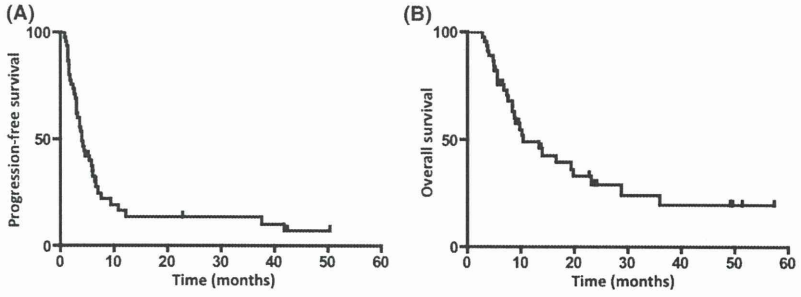

Fig. 2. Progression-free survival (A) and overall survival (B) of urothelial cancer patients treated with paclitaxel, ifosfamide, and nedaplatin as second-line chemotherapy.