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Absence of p53 Overexpression and Favorable Response to Cisplatin-based Neoadjuvant Chemotherapy in Urothelial Carcinomas

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It has been controversial whether cancer cells harboring loss or inactivation of the tumor suppressor p53 are resistant or sensitive to DNA-damaging agents including cisplatin and doxorubicin. Overexpression of mdm2 oncoprotein, a negative regulator of p53, is assumed to be an alternative to p53 dysfunction. Archival urothelial carcinoma specimens obtained from 60 patients prior to cisplatin-based chemotherapy were immunohistochemically studied for overexpression of p53 and mdm2. Thirty-two patients (group I) were treated with chemotherapy in the neoadjuvant setting, while 28 patients (group II) underwent chemotherapy for distant metastases or inoperable locoregional tumors. In group I, the responsiveness was correlated with staining status of p53 (*P*=0.0225) and the combination of p53 and mdm2 (*P*=0.0497). Negative staining of p53 and negative for both p53 and mdm2 could have predicted favorable response to chemotherapy in 16 of 18 (88.9%) and in 12 of 13 (92.3%) tumors, respectively. On the other hand, p53-positive and p53 and/or mdm2-positive staining could have predicted poor response only in 7 of 14 (50.0%) and 8 of 19 (42.1%) tumors, respectively. Disease-specific survival of the p53-negative group was significantly superior to that of the p53-positive group (*P*=0.0086). Difference in survival did not become more significant when overexpression of mdm2 was taken into consideration (*P*=0.0456). In contrast, in group II, there was no correlation of responsiveness to chemotherapy or survival with p53- or p53/mdm2-staining status. The patients with urothelial carcinomas negative for overexpression of p53 will benefit from neoadjuvant chemotherapy. From clinical viewpoint, however, p53 status alone or the combination of p53 and mdm2 status is not enough to identify those patients who will not benefit from the treatment.

Key words: Urothelial cancer — Chemosensitivity — p53 — mdm2

Since cisplatin was introduced, chemotherapy has become an important treatment modality for advanced urothelial carcinomas. Response rates to chemotherapy have been increased to 50–70% by cisplatin-based combination chemotherapy.[1, 2] There is, however, no good marker to predict responsiveness in individual cases.

Several lines of evidence suggest that DNA-damaging agents, including cisplatin and doxorubicin or its derivatives, which are the compounds most frequently used to treat urothelial carcinomas, exert their genotoxic effect by inducing apoptosis.[3–5] One of the key proteins that trigger the apoptotic cell death pathway induced by chemotherapeutic agents is p53.[6, 7] Once DNA damage occurs, p53 is induced and arrests cells in the G1 phase to enhance DNA repair[8] or triggers apoptosis to delete cells with DNA damage, although the pathway of p53-mediated apoptosis is largely unclear.

The mdm2 proto-oncogene product, induced by wild-type p53, acts as a negative regulator for p53.[9] It is considered that continuous overexpression of mdm2 results in inactivation of p53. In urothelial carcinomas, overexpression of mdm2, has been reported to occur in 20–30% of cases,[10, 11] although gene amplification is infrequent.[12] Our recent study clearly demonstrated that the combination of p53 alteration with mdm2 overexpression was a better indicator of microinvasion of superficial urothelial carcinomas than p53 alteration alone.[13] As for the apoptosis induced by anti-cancer drugs, the overexpression of mdm2 confers resistance to cisplatin-induced apoptosis on a human glioblastoma cell line.[14]

The results of *in vitro* and *in vivo* studies as well as preliminary clinical investigations have been controversial as regards the relationship between the status of p53 and chemosensitivity.[6, 15–19] In patients with invasive bladder cancer treated with neoadjuvant cisplatin-based chemotherapy, p53 nuclear accumulation is an independent indicator of poor prognosis, although a relationship between responsiveness and p53 status was not demonstrated.[20] Contrary to these results, however, Cote *et al.*[21] quite recently reported that adjuvant chemotherapy for bladder cancer resulted in decreased risk of tumor recurrence and increased chance of surviving only in patients with tumors harboring p53 alterations. They suggested that p53 alterations conferred increased sensitivity to DNA-damaging agents.

This study was conducted to clarify the relationship between chemosensitivity and p53 inactivation in invasive

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urothelial carcinomas. Inactivation of p53 was judged on the basis of not only p53 aberration alone, but also overexpression of mdm2. The results suggest that absence of p53 aberration is a good predictor of favorable response and prognosis only in patients who undergo chemotherapy as a neoadjuvant modality.

MATERIALS AND METHODS

Patients Between 1986 and 1996, 85 patients with transitional cell carcinomas of the urinary tract were treated intravenously with cisplatin-based combination chemotherapy at our institutes. Of them, 60 patients were included in this retrospective study according to the following criteria; 1) no history of chemotherapy or radiotherapy prior to the treatment, 2) an archival tumor specimen taken prior to chemotherapy was available and 3) existence of measurable lesions before chemotherapy. The patient population consists of 2 groups, depending on the role of chemotherapy in the treatment (Table I). Group I consisted of 32 patients (27/bladder, 5/upper urinary tract) who had tumors confined to the primary organ with or without regional lymph node metastases ($T_{2\text{-}4}$, $N_{0\text{-}2}$, M_0) and who underwent 2 cycles (29 patients) or 3 cycles (3 patients) of cisplatin-based neoadjuvant chemotherapy. Of these, 28 patients underwent a radical operation after chemotherapy, while the remaining 4 with bladder tumors in which complete disappearance of tumor was confirmed by biopsy after chemotherapy underwent bladder preservation. On the other hand, group II consisted of 28 patients with unresectable tumors (T_4 or $N_{2\text{-}3\text{-}}$) or with distant metastatic tumors which were treated with 2 to 6 cycles (mean: 2.5 cycles) of cisplatin-based chemotherapy.

Chemotherapy regimen Thirty of 32 patients of group I were treated with a combination of cisplatin (70–100 mg/m^2), methotrexate (30 mg/m^2) and doxorubicin (30 mg/m^2) or epi-doxorubicin (50 mg/m^2), with or without vinblastine (3 mg/m^2) as a neoadjuvant therapy, while a combination of cisplatin (70 mg/m^2), doxorubicin (50 mg/m^2) and cyclophosphamide (500 mg/m^2) was administered to the other 2 patients with moderately impaired renal function (Table I). As for the patients of group II, 17 were treated with a combination of cisplatin, methotrexate and doxorubicin or epidoxorubicin, with or without vinblastine, 10 were treated with a combination of cisplatin, doxorubicin, and cyclophosphamide, and one patient with HCG-producing bladder cancer was treated with a combination of cisplatin and etoposide (Table I) .

Response criteria Responsiveness to chemotherapy was evaluated basically according to the General Rule for Clinical and Pathological Studies on Bladder Cancer[22] and Renal Pelvic and Ureteral Cancer.[23] Briefly, histopathological findings in surgical specimen judged by our hospital pathologists were taken into account for group I. Accordingly, a complete response (CR) in group I was defined as no evidence of viable tumor cells histopathologically. Tumors which showed a 50% or more reduction in the total volume assessed by CT, but still contained viable tumor cells, were classified into partial response (PR). For group II, the responsiveness to chemotherapy was evaluated after the 2nd cycle of chemotherapy. CR was defined as complete disappearance of all evidence of tumor by

Table I. Patients' Profiles

Group I: neoadjuvant cases (*n*=32)
Age: 51–70 (mean±SD: 62.3±5.1)
Sex: 27 male, 5 female
Primary lesion:
bladder tumor: 27, ureteral or renal pelvic tumor: 5
Chemotherapeutic regimen:
methotrexate+cisplatin+doxorubicin or epirubicin±vinblastine: 30
cisplatin+cyclophosphamide+doxorubicin: 2
Group II: locally advanced or distant metastatic cases (*n*=28)
Age: 43–76 (mean±SD: 64.8±7.7)
Sex: 19 male, 9 female
Predominant sites:
lung metastasis: 12, lymph node metastasis 7, bone metastasis: 4, local: 5
Chemotherapeutic regimen:
methotrexate+cisplatin+doxorubicin or epirubicin±vinblastine: 17
cisplatin+cyclophosphamide+doxorubicin: 10
cisplatin+etoposide: 1[a)]

a) HCG-producing bladder cancer.

Table II. Responsiveness to Chemotherapy and Staining Status of p53, mdm2 and Both

	CR	PR	NC	PD	*P*-value[a)]
Group I					
p53− (18)	7	9	1	1	0.0225
p53+ (14)	1	6	6	2	
mdm2− (22)	7	10	4	1	NS
mdm2+ (10)	1	5	3	1	
p53− & mdm2− (13)	6	6	0	1	0.0497
p53+ or mdm2+ (19)	2	9	7	1	
Group II					
p53− (14)	3	4	4	3	NS
p53+ (14)	0	9	3	2	
mdm2− (22)	3	11	6	2	NS
mdm2+ (6)	0	2	1	3	
p53− & mdm2− (12)	3	4	4	1	NS
p53+ or mdm2+ (16)	0	9	3	4	

a) [CR+PR] vs. [NC+PD] by Fisher's exact test.
NS: not significant.

physical examination, X-rays, radionucleotide scans, CT scans and sonography. PR is 50% or more reduction in the summed products of the longest perpendicular diameters of all measured lesions as evaluated with imaging techniques. NC is 50% or less reduction or no more than 25% increase in the summed products of the longest perpendicular diameters of all measured lesions. PD is 25% or more increase in the summed products of the longest perpendicular diameters of all measured lesions.

Immunohistochemistry p53 status and mdm2 status of tumor specimens were examined prior to the chemotherapy. For distant metastatic cases in group II, tumor samples of the primary lesion substituted for the major target lesions. Specimens were fixed with 10% neutral formalin, embedded in paraffin and stored until the time of examination. A 3 μm section of each archival tumor specimen was placed on a silanized slide. An anti-p53 mouse monoclonal antibody, PAb 1801 (Oncogene Science, Inc., New York, NY) and an anti-mdm2 mouse monoclonal antibody, PAb IF-2 (Oncogene Science, Inc.) at the dilution of 1:100 were used for immunohistochemical staining of both molecules. Staining procedures were the same as previously described.[13)] Five hundred tumor cells in one specimen were examined for staining status and tumors in which more than 10% of the neoplastic cells showed intense, homogenous or heterogeneous nuclear staining were defined as being positive for both p53 and mdm2. Results of immunostaining were assessed by 2 investigators (E. Ö. and Y. K.) independently and average percentage of positive staining was adopted as a final value in each sample.

Statistical analysis Fisher's exact test was used to determine whether the nuclear overexpression of p53 or mdm2 was associated with responsiveness to chemotherapy. The Kaplan-Meier method was used to derive the disease-specific survival curves of the patients with or without overexpression of p53 and mdm2, and the logrank (Mantel-Cox) test was used for comparison of the groups.

RESULTS

There were only two tumors in which judgement of immunostaining by the two investigators differed. Both tumors contained small numbers of cells staining positively for p53 at a frequency around the cut-off point, but were classified into the negative staining group because the mean values of % positivity were lower than 10%. Consequently, the positive nuclear accumulation of p53 was detected immunohistochemically in 29 of 60 (48.3%) tumors. Nuclear overexpression of mdm2 was found in 15 of 60 (25.0%), although the frequency of gene amplification was not determined. In 9 of 60 (15.0%), overexpression of both p53 and mdm2 was found.

The overall response rate (CR+PR/all) to the chemotherapy in group I was 71.9% and that in group II was 57.1%, as shown in Table II. In group I, CR was more frequently observed in the tumors negative for p53 and mdm2 than in positively staining tumors. The responsiveness (CR+PR vs. NC+PD) was correlated with p53-negative and p53/mdm2-negative staining status (P=0.0225 and 0.0497, respectively). Consequently, negative staining for p53 and p53/mdm2 could have predicted favorable response to neoadjuvant chemotherapy in 16 of 18 (88.9% with 95% confidence interval between 65.3 and 98.6%) and in 12 of 13 [92.3% (95% CI: 64.0–99.8)] tumors,

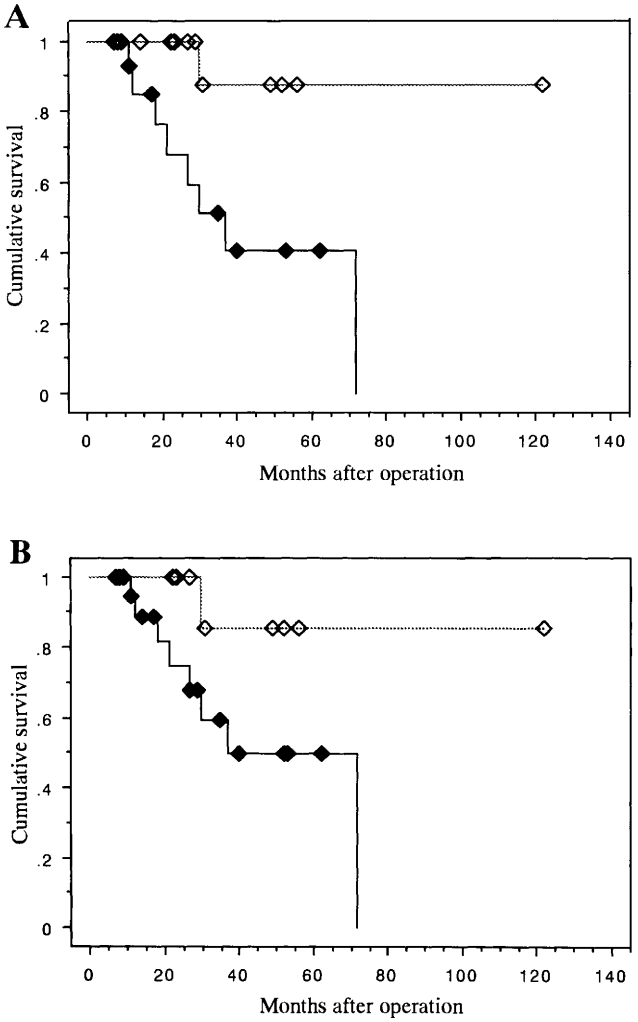

Fig. 1. Kaplan-Meier plots of disease-specific survival for patients of group I in relation to p53-staining status (A) and p53/mdm2-staining status (B). ◆ positive, ◇ negative. A, ◆ *n*=14, ◇ *n*=18, *P*=0.0086; B, ◆ *n*=19, ◇ *n*=13, *P*=0.0456.

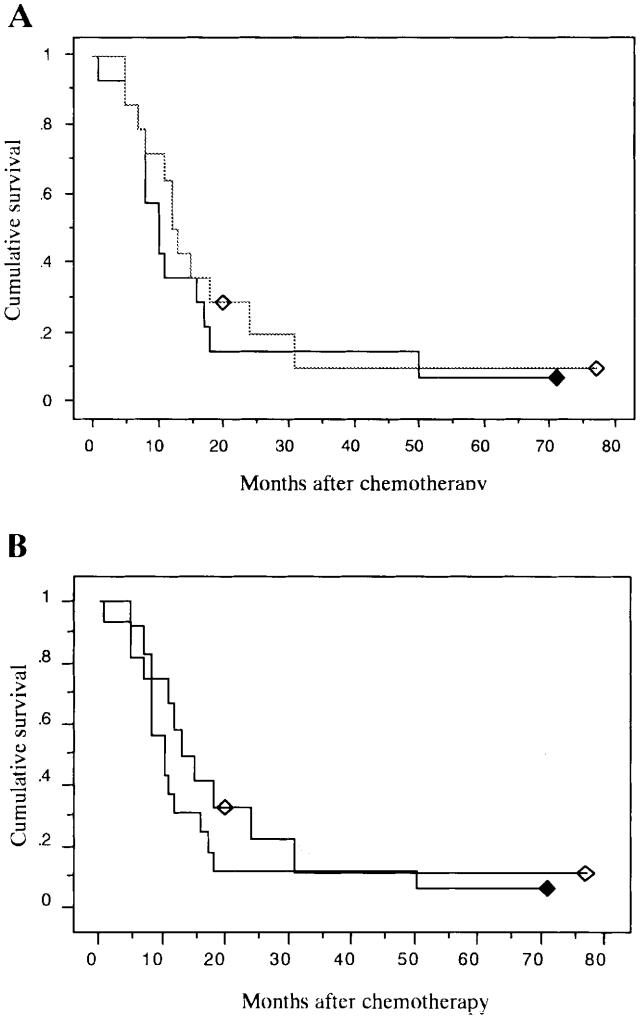

Fig. 2. Kaplan-Meier plots of disease-specific survival for patients of group II in relation to p53-staining status (A) and p53/mdm2-staining status (B). ◆ positive, ◇ negative. A, ◆ *n*=14, ◇ *n*=14; B, ◆ *n*=16, ◇ *n*=12.

respectively. Conversely, p53-positive and p53- and/or mdm2-positive staining could have predicted poor response only in 7 of 14 [50.0% (95% CI: 23.0–77.0)] and 8 of 19 [42.1% (95% CI: 20.3–66.5)] tumors, respectively. On the other hand, in group II, responsiveness to chemotherapy was not associated with p53- or mdm2- or p53/mdm2-staining status, although all 3 CR cases were negative for both p53 and mdm2 in the primary tumors.

In group I, with a median follow-up of 28 months (varied from 7 to 122 months), there were 8 cancer-deaths among the 14 patients with p53-positive tumors, whereas there was only one cancer-death among 18 patients with p53-negative tumors. There was no statistically significant difference in tumor grade, T-stage or node status between the positive and the negative staining groups (data not shown). Responsiveness to chemotherapy in the 8 cancer-death patients with p53-positive tumors was 5 NCs, 1 PD and 2 PRs, while that in the patient who died with p53-negative tumors was PR. The disease-specific cumulative survival curves in the patients with p53-negative tumors were superior to those in the patients with p53-positive tumors (*P*=0.0086), as shown in Fig. 1A. Among the 8 cancer-death patients with p53-positive tumors, 4 patients showed positive staining for both p53 and mdm2. Five patients with mdm2-positive but p53-negative tumors have been alive without recurrence up to now. Therefore, the

difference in the rates of survival did not become more significant when overexpression of mdm2 was taken into consideration together with p53 inactivation (P=0.0456), as shown in Fig. 1B.

In group II, with a median follow-up of 11.5 months (maximal follow-up of 77 months), 25 of 28 patients died of cancer. Two of the 3 living patients (20 months with recurrence and 77 months without evidence of disease after chemotherapy) were negative for p53 and mdm2, while the remaining one (70 months without evidence of disease after chemotherapy) was positive for p53. The rates of survival were not different either between p53-positive and -negative or between p53/mdm2-positive and -negative groups (Fig. 2, A and B).

DISCUSSION

P53 products altered by mutation or complex formation with other proteins have a half-life 4 to 20 times longer than that of the wild-type p53 protein. There is evidence that positive nuclear staining in tumor cells reflects the presence of mutated p53 protein.[24, 25] With the rapid development of a range of p53 protein antibodies which work in fixed tissue, it is now possible to carry out large retrospective studies of the protein expression in a variety of human cancers. False-negative results of immunohistochemistry may be generated as a consequence of premature stop codons, deletions or insertions. The monoclonal antibody PAb 1801, which was used in this study, recognizes an epitope localized between amino acids 40 and 65.[26] Most of the genetic alterations observed in urothelial cancers affect the central region of the gene, from the fifth to the eighth exon. Therefore, PAb 1801 is effectively reactive with not only wild-type p53, but also mutated type p53. In addition, it has good ability to detect nuclear accumulation of p53 even in formalin-fixed, paraffin-embedded tissues when compared to the other antibodies, although false-negative rates of 15 to 20 percent have been reported in bladder cancer.[27, 28]

High-grade and advanced-stage urothelial carcinomas, which are candidates for cisplatin-based chemotherapy either preoperatively or postoperatively, often harbor p53 aberration.[29, 30] Therefore, it is imperative to clarify whether the urothelial carcinomas with p53 aberration are insensitive to or rather sensitive to the current cisplatin-based chemotherapy. The present results strongly suggest that patients with localized urothelial carcinomas that are negative for p53-staining can be expected to respond to neoadjuvant chemotherapy better, and are more likely to benefit from the treatment, than patients with p53-positive tumors, although this result is based on a retrospective investigation of a relatively small number of patients. Sarkis *et al*.[20] also reported that positive nuclear staining of p53 was an independent predictor of poor prognosis in patients with invasive bladder cancer treated with cisplatin-based combined chemotherapy (M-VAC) administered in the neoadjuvant setting. One simple explanation for these results is that tumors with functionally normal p53 are more sensitive to cisplatin-based chemotherapy than those with p53 aberration. Another possibility is that p53-negative urothelial tumors might be less susceptible to metastatic spread during the peri-operative periods, so that complete surgical eradication is more probable than with the p53-positive tumors. On the other hand, the present study suggests that the patients with localized tumors that are positive for p53-staining are less likely to benefit from neoadjuvant chemotherapy than those with p53-negative tumors. It is very difficult to propose an alternative treatment strategy for this subset of patients. At least, radical surgery should be conducted without delay for them. It remains unclear whether radiation therapy is more effective for tumors with p53 aberration.

In the present study, p53 and/or mdm2 staining status was not correlated with chemosensitivity in patients with far-advanced or distant metastatic tumors, although all of the 3 CR cases were p53/mdm2-negative tumors. Cumulative molecular changes in species other than p53 might play a more dominant role in the chemosensitivity of extensive/metastatic diseases. It is very important to find out what genetic alterations are critical in determining chemosensitivity in far-advanced or distant metastatic tumors. As for chemosensitivity and p53 status, Cote *et al*.[21] recently reported the strikingly contrasting result that adjuvant chemotherapy for bladder cancer resulted in decreased risk of tumor recurrence and an increased chance of survivng only in patients with tumors harboring p53 alterations. It is unlikely that the discrepancy is due to the chemotherapeutic agents used, since they used almost identical agents to those used in this study. It is imperative to clarify in a larger-scale prospective study whether the apparent difference as to the effect of p53 dysfunction on chemosensitivity is real, or due to defferences in methodology.

This is the first report in which the overexpression of mdm2 has been taken into account in investigating the relationship between chemosensitivity and dysfunction of p53 in clinical tumor samples. The reported frequency of mdm2 overexpression in human bladder cancer is 20–30%.[11–13] We have recently reported that nuclear overexpression of p53 in combination with mdm2 was more significantly associated with degradation of the basement membrane of superficial bladder tumors than p53-positive staining alone.[13] In contrast, the presence or absence of mdm2 overexpression was not adjunctive to p53 staining status with respect to the prediction of responsiveness to chemotherapy in the present study. Cordon-Cardo *et al*.[31] reported very poor survival for patients with soft tissue sarcomas simultaneously positive for p53 alteration and

mdm2 overexpression. They speculated that mutant p53-mdm2 protein complexes might gain additional functions that are more damaging than that of mutant p53 alone or wild-type p53-mdm2 complex. We, however, found no association with poorer response to chemotherapy or survival for patients with tumors positive for both p53 and mdm2, although the number of such tumors was small (9 of 60, 15%).

In conclusion, the present study clearly indicates that patients with p53-negative staining will benefit from neoadjuvant chemotherapy. On the other hand, evaluation of the status of p53 alone or p53/mdm2 is not enough to predict those patients who will not benefit from the treatment.

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