Advances in Brief

Molecular Determination of Perivesical and Lymph Node Metastasis after Radical Cystectomy for Urothelial Carcinoma of the Bladder

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Abstract

Purpose: **Current methods used to determine the pathological stage of the primary tumor and associated lymphatics after radical cystectomy are tedious, costly, and may lack the sensitivity afforded by molecular approaches such as reverse transcription-PCR (RT-PCR) for markers specific for urothelial tissue such as the *uroplakin II* (*UPII*) gene. Thus, we sought to evaluate an objective and sensitive molecular approach for the assessment of perivesical extension and lymph node status after radical cystectomy, based on the detection of *UPII* expression using RT-PCR and compare this assay to standard clinical and pathological examination.**

Experimental Design: **From November 1999 to September 2000, 27 patients with clinical T_a–$T_3N_0M_0$ urothelial bladder cancer underwent radical cystectomy, 19 (70%) of which also had pelvic lymphadenectomy. At the completion of cystectomy, systematic biopsies of the external surface of the bladder specimen as well as from the largest palpable lymph node found at lymphadenectomy were obtained for molecular analysis. RT-PCR analysis for *UPII* mRNA was carried out on these biopsy specimens, and results were compared with data obtained from conventional pathological examination.**

Results: **Pathologically organ-confined tumors had a 42% (5 of 12) incidence of positive signals in the perivesical tissues and 17% (1 of 7) in the lymph nodes. Corresponding percentages for $pT_{3a}N_0$ and pT_{3b}–T_4N_0 lesions were 67% (4 of 6)/25% (1 of 4) and 67% (4 of 6)/33% (2 of 6), respectively. Overall, pathologically node-negative cancers had a perivesical positivity rate of 54% (13 of 24) and a lymph node positivity rate of 25% (4 of 16). All patients with pathologically positive nodes had positive *UPII* signals in the lymph node sample.**

Conclusions: **This molecular assay aimed at assessing perivesical extension and lymph node status after radical cystectomy appears to identify patients that may harbor residual disease not appreciated by conventional histology. Larger studies with 5–7-year follow-up will be required to determine the prognostic significance of such molecular information.**

Introduction

Pathological stage and lymph node status are the common primary prognostic factors after radical cystectomy or cystoprostatectomy for urothelial (transitional) bladder cancer. Survival data from several series stratifying patients by pathological tumor stage using the 1992 UICC[2]/AJCC classification (1) indicate that $pT_{2–3a}$ patients have a 50–65% 5-year survival (Table 27.6 in Ref. 2). In contrast, patients with pT_{3b-4} tumors only have a 10–35% 5-year survival (Table 27.7 in Ref. 2). In addition, the incidence of positive pelvic lymph nodes is also a function of pathological stage ranging from <8% in pT_{a-1} to 10–30% in $pT_{2–3a}$ and 27–64% in pT_{3b-4} (Table 27.9 in Ref. 2). Five-year survival of patients with pathologically proven pelvic lymphatic metastases have ranged from 0 to 36% (Table 27.11 in Ref. 2). Two studies (3, 4) have examined the relationship of the pathological stage of the primary tumor to survival in patients with positive nodes and have found this variable to be a strong predictor of survival. Thus, accurate determination of the pathological stage including lymph node status is critically important for deriving clinically useful information after radical cystectomy.

However, the detection of pathological stage including surgical margins and perivesical extension after radical cystectomy in bladder cancer can be cumbersome in view of the size of the specimen. This leads pathologists to focus and examine microscopically only areas of visible or palpable tumor. Because up to 15% of bladder specimens (5) do not harbor a detectable primary lesion (*i.e.*, stage pT_0), this further compromises the accurate evaluation of pathological stage. In addition, thorough examination of the excised lymphatic tissues adds to the time and expense required for the complete pathological staging of the surgical specimen. Finally, because not all excised tissue is examined microscopically, the potential for understaging does exist. These limitations as well as the desire to enhance sensitivity of staging have led to the search for an objective, rapid, reproducible, and simple assay to determine the status of surgical margins and lymph node status after radical cystectomy.

Our approach was to use RT-PCR for *UPII* for the analysis of tissue samples taken from the exterior of the excised radical

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[2] The abbreviations used are: UICC, Union International Contre Cancer; AJCC, American Joint Committee on Cancer; RT-PCR, reverse transcription-PCR; UPII, uroplakin II; TNM, Tumor-Node-Metastasis.

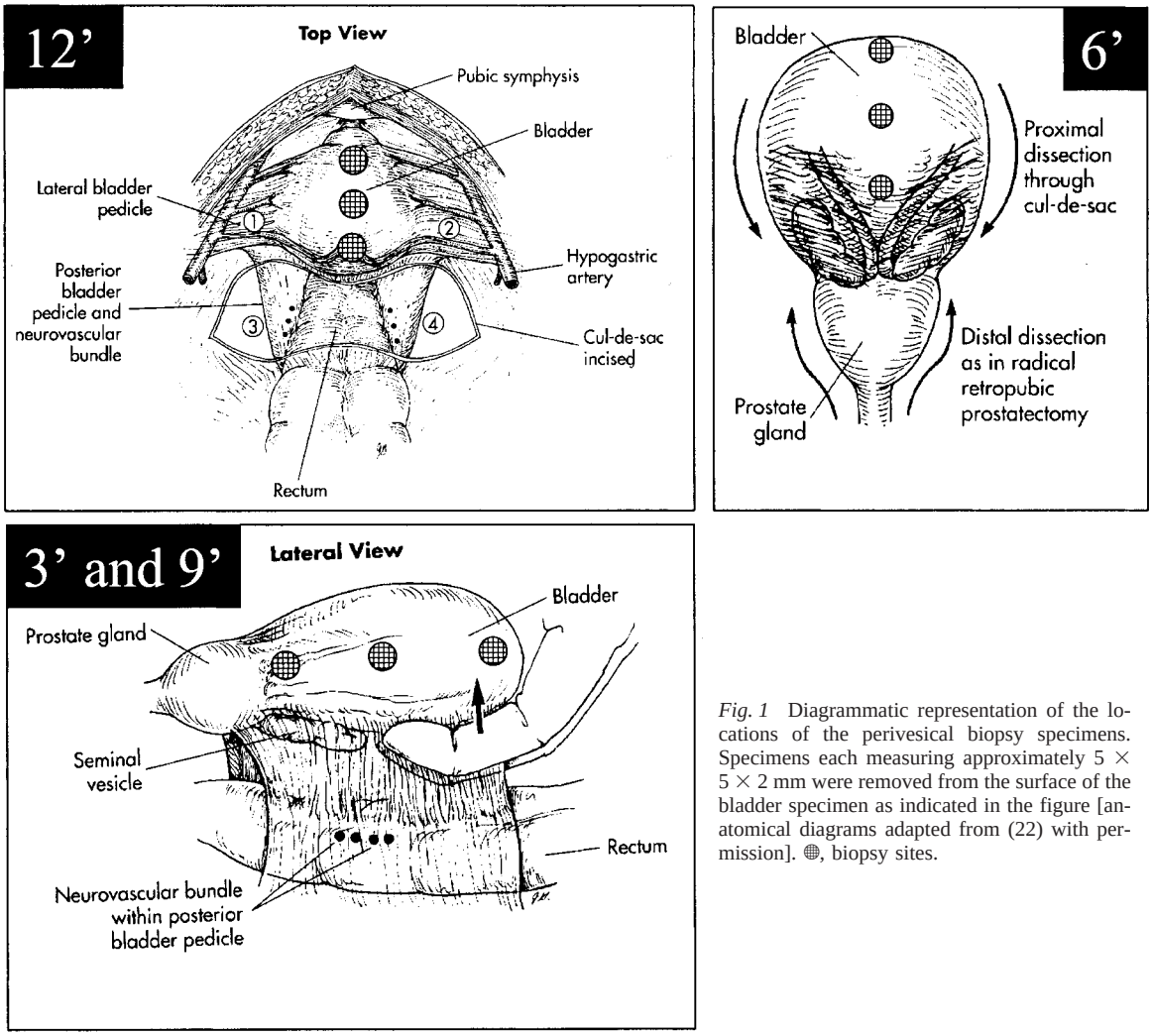

Fig. 1 Diagrammatic representation of the locations of the perivesical biopsy specimens. Specimens each measuring approximately 5 × 5 × 2 mm were removed from the surface of the bladder specimen as indicated in the figure [anatomical diagrams adapted from (22) with permission]. ◍, biopsy sites.

cystectomy specimen and from the largest palpable lymph node. Uroplakins are specific differentiation products of terminally differentiated superficial urothelial cells (6). Several recent studies have examined their expression in urothelial cancers either immunohistochemically (7, 8) or using RT-PCR techniques (9–11). In any given urothelial tumor tissue sample, despite heterogeneity, uroplakins are almost always expressed to some degree. By tailoring the RT-PCR technique (*i.e.*, nested *versus* nonnested) to the clinical question, this assay can be designed to detect (nested) or not detect (nonnested) *UPII*-positive cells in the circulating blood of patients with no clinical evidence of metastatic bladder cancer. Thus, by applying the nonnested technique, even significant blood contamination of our tissue specimens will not induce false positivity of the assay because all our patients are clinically free of metastatic disease. Herein we report our experience with this nonnested RT-PCR technique (11) and compare *UPII* expression in these perivesical and lymphatic tissues excised at radical cystectomy to conventional histopathological assessment of tumor stage and lymph node status.

Materials and Methods

Patient Population and Surgical Procedures

From November 1999 through September 2000, 27 patients with clinical stage pT_a–$T_3N_0M_0$ underwent radical cystectomy for bladder cancer at the University of Virginia Health Sciences Center and at the University of Western Ontario Health Sciences Center. No selection criteria were applied to exclude or include participation in this study. Clinical stage was assigned using the 1997 AJC/UICC TNM System (12). All patients had computed tomography of the abdomen and/or i.v. pyelography and pathological review of their biopsies (if done outside the two participating institutions) before surgery. All patients underwent radical cystectomy with (19 of 27) or without (8 of 28) pelvic lymphadenectomy. The 3 of 9 patients who did not have pelvic lymphadenectomy had received prior bladder irradiation for bladder cancer treatment, and lymphadenectomy was deemed technically impossible, whereas in the remaining 6 cases, the harvesting was omitted because of the lymph node specimens having no distinct palpable nodes. This prospective

Table 1 Patient and tumor characteristics

		TUR[a] specimen		Cystectomy specimen		RT-PCR results (UPII)[c]	
No.	Age (yr)	Clin stage[b]	Clin grade	Path stage[b]	Path grade	Perivesical tissues	Lymph node
1	76	T_1	3	T_0N_0	NS	0/5	0/1
2	78	T_1	3	T_0N_0	NS	0/5	NS
3	65	T_2	3	T_0N_0	NS	1/5	NS[d]
4	47	T_2	2	T_aN_0	3	2/5[e]	1/1
5	43	T_2	2	T_1N_0	1	0/4	NS
6	77	T_1	3	T_1N_0	3	0/5	0/1
7	67	T_1	2	T_1N_0	3	4/5[e]	0/1
8	68	T_a	3	T_2N_0	3	4/4	NS[d]
9	64	T_1	3	T_2N_0	3	0/5	0/1
10	75	T_2	3	T_2N_0	3	2/5	0/1
11	58	T_2	2	T_2N_0	2	0/4	NS
12	80	T_2	3	T_2N_0	3	0/5	NS
13	69	T_3	3	$T_{3a}N_0$	3	1/5	0
14	66	T_2	2	$T_{3a}N_0$	3	3/5	1/1
15	63	T_2	3	$T_{3a}N_0$	3	0/5	0/1
16	62	T_3	3	$T_{3a}N_0$	3	5/5	0/1
17	82	T_2	3	$T_{3a}N_0$	3	0/4	NS[d]
18	79	T_1	3	$T_{3a}N_0$	2	5/5	NS
19	51	T_3	3	$T_{3b}N_0$	3	4/4	0/1
20	67	T_1	3	$T_{3b}N_0$	3	4/4	0/1
21	74	T_2	3	$T_{3b}N_0$	3	4/4	1/1
22	77	T_3	3	$T_{3b}N_0$	3	2/4	1/1
23	80	T_2	3	$T_{3b}N_0$	3	0/5	0/1
24	74	T_3	3	$T_{4a}N_0$	3	0/5	0/1
25	49	T_2	2	T_0N_1	NS	0/5	1/1
26	62	T_3	3	$T_{2a}N_1$	3	0/5	1/1
27	71	T_3	2	$T_{4a}N_2$	2	3/5	1/1

[a] TUR, transurethral resection; Clin, clinical; Path, pathological; NS, no specimen.
[b] Staged using 1997 UICC/AJC manual (12).
[c] Number of +*UPII* samples/total RT-PCR samples. All biopsies from one side (Fig. 1) were preserved in the same vial of RNALater (Ambion) and counted as 1 sample.
[d] No lymphadenectomy carried out because of prior radiotherapy for bladder cancer.
[e] Shown in Fig. 2.

study was approved by the Institutional Review Boards of both participating centers.

Specimen Processing and Biopsy Sample Collection

Patients with Urothelial Bladder Cancer. All radical cystectomy specimens were processed in a similar fashion and pathological stage assigned according to the 1997 AJC/UICC TNM System (12). After complete excision of the radical cystectomy specimen from the patient, the specimen was placed on the side table where two to three samples/side, each measuring approximately 5 × 5 × 2 mm, were removed from its surface, as shown in Fig. 1. In addition, similarly sized samples were removed from the bladder surface overlying any palpable intravesical tumor. All biopsy samples from one side (Fig. 1) were preserved in the same vial and counted as 1 sample (Table 1). Once the bilateral lymphadenectomy was completed, a 1-mm-wide cross-section from the largest and/or most clinically suspicious (for metastatic disease) samples was removed. In all cases, this sampled lymph node was included in the final histopathological analysis. Thus, a maximum of 5 perivesical samples and 1 lymph node sample were available for each patient. All tissue samples were rinsed twice in sterile saline and then placed in 1 ml of RNALater (Ambion) and maintained on crushed ice and/or 4°C until placed in −80°C 3–48 h later.

Patients with Non-Urothelial Cancers. To evaluate the specificity of the *UPII* RT-PCR, we also evaluated patients with pelvic malignancies using similar methodologies. Two patients with colon cancer and 2 patients with adenocarcinoma of the prostate were used as such "controls." Tissue samples were rinsed twice in sterile saline and then placed in 1 ml of RNALater (Ambion) and maintained on crushed ice and/or 4°C until placed in −80°C 3–48 h later.

RNA Isolation and RT-PCR

Surgical samples were removed from the RNALater solution and homogenized in excess Trizol (Life Technologies, Inc.) reagent using a PCR tissue homogenizer (Omni International, Madison, WI) with disposable blades, which eliminates the cross-contamination of tissue samples during isolation procedure. RNA quantification was determined spectrophotometrically using a Beckman DU-50 spectrophotometer (Beckman). Three hundred ng of total RNA were used as a starting material for each reaction, except the *UPII* positive (RT4 bladder cancer cell RNA) where 10 ng were used. The primers used in RT-PCR for *UPII* amplification were as described by Li *et al.* (11). Li *et al.* (11) found that the technique was able to detect 1 *UPII*-positive RT4 bladder cancer cell line (see below) in 5 ml of normal human blood. Using this technique, no patients with

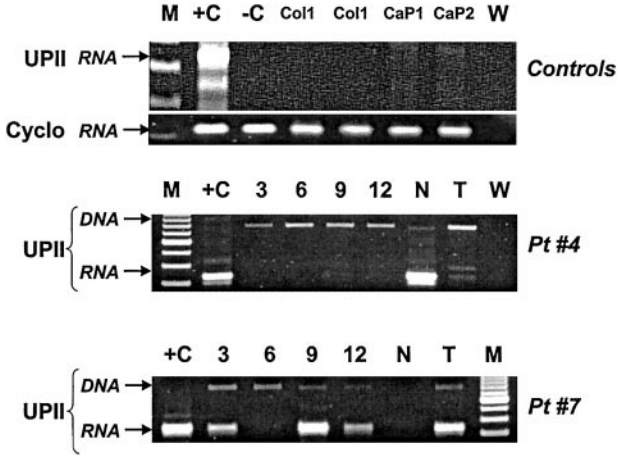

Fig. 2 RT-PCR reactions. RT-PCR was performed for *UPII* as described in "Materials and Methods." The final reaction was separated electrophoretically and visualized with ethidium bromide. Representative PCRs are shown above. *Lane M,* marker lane; *Lanes 3, 6, 9,* and *12,* "clock" positions of bladder biopsies corresponding to those shown in Fig. 1; *Lane T,* the perivesical biopsy overlying palpable tumor if present; *Lane N,* lymph node sample; *Lane W,* distilled H_2O control; *Lane +C,* RT4 RNA that served as positive control; *Lane −C,* LnCaP RNA, which served as negative control. *Cyclo,* cyclophilin; *Controls,* data obtained on patients with non-urothelial pelvic tumors; *Col 1* and *Col 2,* samples from patients with colorectal adenocarcinoma; *CaP1* and *CaP2,* samples from patients with prostate adenocarcinoma; *Pt #4,* patient 4 (see Table 1 for patient data) shown was UPII positive in the lymph node specimen despite having a histopathological stage of pT_aN_0; *Pt #7,* patient 7 shown was UPII positive in several bladder specimen biopsies while having a histopathological stage of pT_1N_0.

clinically localized disease, as is the case for our patient cohort, had positive signals in peripheral blood, which obviated the need and expense of parallel analysis of blood samples in our study (11). RT-PCR for *UPII* carried out using Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's recommendation. The RT-PCR conditions were compared between the program (described below) used in the current study and the earlier report (11) using the above kit in a GeneAmp PCR System 9700 (PE Applied Biosystems) machine. On the basis of the comparison, the following program was used to analyze the samples. The continuous thermal cycles were as follows: 50°C for 30 min, 95°C for 15 min, and then 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min.

Cyclophilin message was amplified by RT-PCR to evaluate the quality and integrity of the extracted mRNA in 2 patients with a completely negative *UPII* RT-PCR because this gene is ubiquitously expressed in all human cells (13). Detection of cyclophilin expression was also carried out using the same as above OneStep RT-PCR reagent at the following conditions: 50°C for 30 min, 95°C for 15 min, and then 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min. All products were resolved by horizontal gel electrophoresis (1% agarose, 0.5× Tris acetate, 0.04 M Tris acetate, and 0.5 mM EDTA) using 20% of the reaction product. Both patients had positive cyclophilin RT-PCR signals in all extracted tissue samples (data not shown), indicating that lack of *UPII* positivity was not attributable to RNA degradation.

Cell Lines and Culture Conditions

The human bladder cancer cell line RT4 was used as a positive control, whereas the human prostate cancer cell line LNCaP was used as a negative control, as described previously (11). The LNCaP and RT4 cells were grown in T and DMEM F12 medium, both supplemented with 5% fetal bovine serum (Life Technologies, Inc.), respectively (14). Total cellular RNA for RT-PCR was isolated from almost confluent cell cultures using Trizol (Life Technologies, Inc.) as per the manufacturer's recommendations for adherent cell lines.

Results

Patient Demographics and Tumor Stages. The characteristics of the 27 tumors, including demographic information and clinical/pathological stages, are shown in Table 1. The patient cohort consisted of 9 women and 17 men with an average age of 68 ± 11 years. Eight of 27 (30%) patients had cystectomies for clinically superficial (T_a, T_{is}, T_1) cancers, whereas 19 of 27 (30%) patients had muscle-invasive neoplasms. Of the patients with superficial disease, 2 of 8 (25%) had no evidence of tumor in the radical cystectomy (*i.e.,* pT_0), and no patients had node-positive disease (pN_1). Of the patients with muscle-invasive disease, 2 of 19 (10%) had no evidence of tumor in the radical cystectomy (pT_0), whereas 3 of 19 (16%) patients had node-positive disease ($pN_{1–3}$). Overall, 1 of 4 (25%) patients with pT_0 disease had lymph node-positive disease.

Detection of *UPII*-expressing Cells in Perivesical and Nodal Tissue Samples. In every reaction, the LNCaP cell line was used as a negative control, and human bladder cancer cell line RT4 was used as a positive control (Fig. 2). In addition, water was used as the negative control because well reactions were set up with or without any reverse transcriptase and kept at 4°C until the denaturation starts to test for primer and/or procedure-generated artifact, PCR carry-over, or laboratory contamination of the *UPII* template. Finally, an expected sized product of 322 bp (Fig. 2) was cloned, sequenced, and confirmed a homology of 100% with *UPII* gene sequence.

Using this approach, we carried out RT-PCR for *UPII* on all perivesical and nodal tissue samples collected after the removal of the cystectomy specimen. A total of 154 specimens were examined by RT-PCR for *UPII,* of which 44 (29%) were positive for *UPII*. In terms of individual biopsy specimens, 44 of 126 (35%) of the perivesical tissue specimens were positive, whereas 7 of 19 (37%) of the lymph node tissue specimens had detectable *UPII* signals. The median number (range) of positive perivesical tissue specimens in these 14 patients was 3.5 (1–5).

Comparison of RT-PCR Results on Perivesical and Nodal Tissue with Conventional Pathological Stage. The results of RT-PCR for the *UPII* gene product in comparison to the clinical and pathological features of the radical cystectomy specimens are shown in Tables 1 and 2. In the 8 patients who had radical cystectomy for clinically superficial disease (cT_a–T_1), 4 (50%) had at least one perivesical biopsy positive for *UPII* expression (Tables 1 and 2), but no positivity was seen in the nodal tissues. In 19 patients with clinically invasive disease (cT_2–T_4), 10 (53%) had at least one perivesical biopsy positive for *UPII* expression and 7 of 14 (50%) had positivity in the

Table 2 Comparison of clinical data and conventional pathological features of the radical cystectomy and lymphadenectomy specimens with RT-PCR results

	No. of patients having cystectomy		No. of patients having lymphadenectomy			
Tumor stage[a]	*n*	*UPII*+[b]	*n*	*UPII*+[b]	%+margin	%+Node
cT_a–T_1N_0	8	4	5	0	50%	0%
cT_2–T_4N_0	19	10	14	7	53%	50%
$pT_0/T_a/T_1/T_2N_0$	12	5	6	1	42%	17%
$pT_{3a}N_0$	6	4	4	1	67%	25%
pT_{3b}–T_4N_0	6	4	6	2	67%	33%
Total pN_0	24	13	16	4	54%	25%
Total pN+	3	1	3	3	33%	100%
Total	27	14	19	7	52%	37%

[a] Staged using 1997 UICC/AJC manual (12).
[b] Number of patients with any *UPII*-positive samples.

lymph node specimens. Overall, 14 of 27 (52%) and 7 of 19 (37%) patients had positive *UPII* biopsies in perivesical or lymph node samples, respectively.

Correlation of pathological staging with molecular analysis for *UPII* expression reveals a trend toward increased frequency of *UPII* positivity in both the perivesical and lymph node biopsies as a function of pathological stage in patients with pathologically negative lymph nodes (Table 2). In addition, a trend toward a higher number of *UPII*-positive perivesical biopsies per patient as a function of stage was also present (Fig. 3). All 3 patients with nodal involvement had *UPII* positivity in the nodal sample, whereas only 1 (33%) had any positive perivesical biopsies. Interestingly, 1 of 12 (17%) patients with pathologically organ-confined, node-negative disease had positive lymph node *UPII* signals, whereas 3 of 12 (25%) patients with pT_3–T_4N_0 disease had this finding.

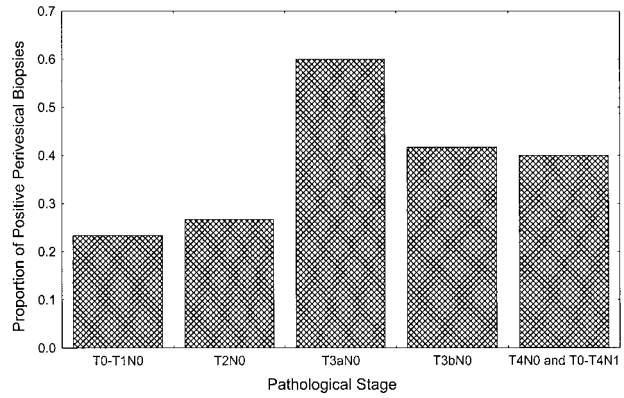

Fig. 3 Histogram representing the proportion of perivesical biopsy samples (1 sample per side and 1 overlying tumor containing multiple tissue specimens as described in "Materials and Methods" and Fig. 1) expressing *UPII* as a function of pathological stage. The denominator used was the number of perivesical biopsy specimens extracted for RNA.

Discussion

Two large studies have examined the impact of the number and size of positive nodes on the survival of patients after radical cystectomy and found a trend toward decreased survival in patients with increasing bulk of positive nodes (3, 4). Interestingly, the differences in survival between groups with minimal involvement [*i.e.*, 1–5 positive nodes or pN_1 (3)] to those with more significant involvement were small. This observation serves to highlight that despite radical surgery, any nodal positivity is a dire prognostic factor. However, it has also been recognized for some time that patients with metastatic disease limited to the lymphatics may have a better prognosis compared with those harboring metastases at other sites (15, 16). This finding, coupled with the advent of novel chemotherapeutic agents with less toxicity than classic regimens (17, 18), offer the possibility for significant improvements in prognosis in patients with locally advanced or with pelvic lymph node metastases treated in an adjuvant setting (19).

Because there are limitations to the sensitivity of detecting perivesical extension and lymph node positivity in patients undergoing radical cystectomy, we sought to devise a technique that could improve on this. Thus, we proposed to biopsy the surface of the primary specimen once removed from the patient and in addition, a representative cross-sectional slice from the largest and/or most suspicious lymph node and analyze all samples using molecular techniques. This sampling procedure is simple, rapid, and safe for the patient, incurring no penalty in terms of morbidity or operative time. However, before discussing this technique any further, it must be clearly emphasized that the results presented here are clearly done both as "proof of principle" and to be hypothesis generating for further studies. They are very preliminary and thus any clinical relevance or significance of these results is purely hypothetical and speculative at this point.

Although others have used RT-PCR for *UPII* for the detection of floating urothelial cancer cells in blood (10, 11), the direct examination of bladder surface and lymph node biopsy samples for micrometastatic bladder cancer cells after radical cystectomy has not been described previously. Because of the novelty of this study, we have attempted to methodically eliminate factors that could confound the interpretation of our results. We have attempted to address the issue of blood contamination of the samples in two ways: (*a*) we have taken care to rinse the biopsy samples thoroughly, prior to analysis, to eliminate any gross blood contamination; and (*b*) the RT-PCR tech-

nique selected was one that was designed not to detect circulating urothelial cells in the circulation of patients with clinically localized bladder cancer (11), even when much larger quantities of blood than would be contaminating our specimens were analyzed. Furthermore, in patients with *UPII*-positive biopsies, not all biopsy samples were positive, suggesting that this assay was not artifactually contaminated by tumor cells circulating in the bloodstream. In addition, we found that all histologically positive lymph nodes were detected by the molecular analysis (Table 2). This latter finding suggests that the molecular methodology has a sensitivity at least equivalent to conventional pathological analysis of the nodes.

Only long term follow-up of patients with pathologically node-negative but *UPII*-positive signals in the lymph nodes will reveal whether these patients will have a clinical recurrence. Although infrequently seen in our small series (4 of 16 patients; 25%), this percentage is similar to the rate of recurrence observed for patients with such organ-confined disease (20), suggesting that most patients with organ-confined disease and positive *UPII* expression in the nodes will eventually fail. However, as mentioned earlier, in the absence of long term follow-up, such conclusions are purely speculative. Continued long-term follow-up will also reveal the clinical significance of the molecular positive perivesical biopsies in the context of either clinically or molecularly positive lymph nodes. Because the degree of positivity of these latter biopsies is higher than the expected failure rate after cystectomy (20), it is unlikely that they reflect residual cancer left inside the patient after cystectomy. Instead, they may reflect perivascular permeation of the bladder wall by cancer cells, which by definition is removed at the time of radical cystectomy. However, in view of the fact that pathological stage has been shown to be an important prognostic factor even in patients with clinically positive nodes (21), this finding may conceivably also be the case for the molecular analysis. For example, patients with *UPII* expression in lymph node tissue and extensive expression of *UPII* in most perivesical biopsies (Fig. 3) may have a higher risk of local or metastatic recurrence than a patient with only lymph node *UPII* expression and minimal or absent perivesical positivity.

Finally, because some series have shown a trend toward improved cure rate in patients with minimal nodal disease with radical surgery alone (3), it is conceivable that patients with molecularly positive but histologically negative lymph nodes may have an intermediate prognosis between patients with complete lack of *UPII* expression in the lymph nodes and those with microscopically positive lymph nodes. Conversely, if these patients do fail with a high percentage, they may be in the best prognostic group with respect to potential cure from adjuvant chemotherapy in view of the minimal bulk of disease. As mentioned earlier, in the absence of long-term follow-up, such conclusions are purely speculative.

In conclusion, this RT-PCR-mediated approach appears to detect all clinically positive lymph nodes. In addition, this methodology identifies patients with positive *UPII* expression in microscopically negative lymph node samples. This approach also detects *UPII* expression in perivesical biopsies in 54% of patients with pathologically node-negative disease. Although these very preliminary results are promising because of the close similarity of the lymph node-derived molecular results with eventual clinical failure after cystectomy in large series, only long-term follow-up of these patients will determine the validity and clinical relevance of these findings.

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