Int. J. Cancer: **111,** 934–939 (2004)

Publication of the International Union Against Cancer

DETECTION OF CIRCULATING CANCER CELLS EXPRESSING UROPLAKINS AND EPIDERMAL GROWTH FACTOR RECEPTOR IN BLADDER CANCER PATIENTS

Iman OSMAN[1,2]*, Melissa KANG[2], Andy LEE[2], Fang-Ming DENG[2], David POLSKY[2], Maryann MIKHAIL[2], Caroline CHANG[2], Dexter A. DAVID[3], Nandita MITRA[3], Xue-Ru WU[1,2], Tung-Tien SUN[1,2] and Dean F. BAJORIN[3,4]

[1]*Kaplan Comprehensive Cancer Center, New York, NY, USA*
[2]*New York University School of Medicine, New York, NY, USA*
[3]*Memorial Sloan-Kettering Cancer Center, New York, NY, USA*
[4]*Joan and Sanford Weill Medical College of Cornell University, New York, NY, USA*

Our purpose was to determine the clinical relevance of the detection of circulating tumor cells (CTCs) expressing urothelial and epithelial markers in bladder cancer patients. Sixty-two patients who presented to Memorial Sloan-Kettering Cancer Center between July 2000 and September 2001 were studied. Peripheral blood was tested by nested RT-PCR assay for uroplakins (UPs) Ia, Ib, II and III as well as for epidermal growth factor receptor (EGFR). We determined the sensitivity and specificity of each individual marker and the combinations of UPIa/UPII and UPIb/UPIII. The latter strategy was based on our data, which showed that UPIa and UPIb form heterodimers with UPII and UPIII, respectively. Forty patients had clinically advanced bladder cancer and 22 had no evidence of disease at the time of assay. Eight of the 22 patients recurred during the follow-up period. All 8 patients were positive at presentation for UPIa/UPII. The combination of UPIa/UPII provided the best sensitivity (75%) of detecting CTCs, with a specificity of 50%. The combination of UPIb/UPIII was the most specific (79%) but had modest sensitivity (31%). Detection of EGFR-positive cells alone and in combination with UPs was inferior to that for UPIa/UPII. Combinations of urothelial markers are superior to single urothelial or epithelial markers in detecting CTCs in bladder cancer patients. Further efforts are under way to confirm the potential predictive value of these markers in a prospectively designed study of a larger cohort of patients.

Key words: *bladder cancer; circulating tumor cell; uroplakin*

The development of a better prognostic marker in bladder cancer could improve treatment decisions and refine patient stratification for clinical trials. Randomized trials have shown a survival advantage of using combination chemotherapy before surgery to treat preexisting micrometastases.[1] Despite the fact that only 50% will relapse, this strategy requires that all patients with muscle-invasive TCC receive chemotherapy before surgery. Disadvantages of this approach include significant morbidity due to the side effects of chemotherapy, substantial increase in length of treatment by several months and considerable financial cost of avoidable treatment for 50% of patients.

An alternative to neoadjuvant chemotherapy would be to administer chemotherapy only to patients with a high risk of relapse after surgery. Although more rational, this approach is hindered by the limited prognostic value of pathology alone in bladder cancer. Critical to this approach of selecting patients at highest risk of relapse after surgery is the development of a biomarker(s) that can provide the best sensitivity and specificity for the presence of micrometastatic disease. Detection of CTCs has the potential to fulfill the criteria for a good prognostic marker of relapse. While it is well known that not all CTCs give rise to clinically significant metastatic deposits, it has been demonstrated that patients with persistent seeding of CTCs are more likely to develop clinical metastases and resistance to treatment.[2–4]

Lack of a marker specific for urothelial cancer has been the main obstacle in the detection of CTCs in patients with bladder cancer compared to other solid tumors. However, studies have led to the discovery of UPs, the first urothelium-specific marker, and to the cloning of their cDNAs and genes.[5–7] We have previously shown that the apical surface of differentiated urothelial cells is covered with plaques that contain 4 major integral membrane proteins, UPs Ia (27 kDa), Ib (28 kDa), II (15 kDa) and III (47 kDa).[8,9] Also, we have demonstrated that UPII and UPIII are cross-linked to UPIa and UPIb, respectively, suggesting the existence of 2 types of heterodimers, consisting of UPIa/UPII and UPIb/UPIII.[10,11] As UPs have been shown to be markers of urothelial differentiation by several independent investigators,[12–16] their level of expression might be low in poorly differentiated tumors. Nevertheless, we have shown previously that UPs are decreased at the protein level in such tumors; however, evidence indicates that even very poorly differentiated urothelial cells continue to express UPs at the mRNA level.[17] We also added to our panel an epithelial marker, EGFR, which is associated with undifferentiated basal urothelial cells and overexpressed in primary and metastatic bladder cancer.[18,19]

In the present study, we evaluated the utility of using both UPs and EGFR in detecting CTCs of bladder cancer patients. We reasoned that UPs could be used individually as well as in combinations, taking advantage of the known paired expression among the 4 major types.

MATERIAL AND METHODS

Patient characteristics

Bladder cancer patients who presented for treatment at the Sidney Kimmel Center for Prostate and Urological Cancers at MSKCC between July 2000 and September 2001 were asked to enroll in the study, which was conducted using an institutional review board–approved protocol. All patients who voluntarily enrolled in the study signed an informed consent before enrollment. The staging evaluation included a complete history and physical examination, an automated blood cell count and imaging studies including chest, abdominal and/or pelvic computerized

Abbreviations: AJCC, American Joint Committee on Cancer; CTC, circulating tumor cell; DEPC, diethylpyrocarbonate; EGFR, epidermal growth factor receptor; MAb, monoclonal antibody; MOP, 3-(N-morpholino)-propanesulfonic acid; MSKCC, Memorial Sloan-Kettering Cancer Center; NYUSM, New York University School of Medicine; TCC, transitional cell carcinoma; UP, uroplakin.

*Correspondence to: New York University School of Medicine, 550 First Avenue (H-100), New York, NY 10016, USA. Fax: +212-951-3214. E-mail: iman.osman@med.nyu.edu

Received 6 February 2004; Accepted after revision 25 February 2004

DOI 10.1002/ijc.20366
Published online 20 May 2004 in Wiley InterScience (www.interscience.wiley.com).

topographic scans when clinically indicated. Staging at the time of the initial blood sample collection was defined according to the AJCC guidelines.[20] Data were collected on all clinicopathologic parameters, such as age at operation, gender, location and size of primary tumor, clinical and pathologic status of lymph nodes, presence of extranodal metastases and site(s) of distant metastases. Follow-up continued for the length of the study (median 15 months).

Samples

All blood samples were processed by a methodology developed by a collaborative group of investigators at NYUSM and MSKCC. Blood samples were drawn by routine venipuncture at MSKCC, labeled with a unique identification code devoid of patient identity and transferred to NYUSM within 4 hrs of venipuncture. Blood (8–12 ml) was collected in EDTA tubes and put immediately on ice for transfer to NYUSM. Laboratory personnel and investigators at NYUSM were blinded to patients' demographics, identity and clinical information. Blood was subjected to erythrocyte lysis using hypotonic buffer [10 mM $KHCO_3$, 155 mM NH_4Cl and 0.1 mM EDTA (pH 7.4)]. Leukocytes and CTCs were recovered by centrifugation and washed once with PBS. RNA was extracted using a phenol:chloroform:isoamyl alcohol mixture and following the protocol of the RNAgents Total RNA Isolation System (Promega, Madison, WI). The yield and purity of RNA were determined by spectrophotometry at A_{260}/A_{280} absorbance. RNA (1 μg) was combined with 3 volumes of formaldehyde RNA loading dye (Ambion, Austin, TX) and run on a 1% agarose gel made in 1 × MOPS buffer to assure the quality of RNA by observing intact bands corresponding to the 28 S and 18 S ribosomal RNA subunits. RNA was stored at –70°C in DEPC-treated water until use.

Reverse transcription of mRNA

Total RNA (1 μg) was reverse-transcribed using Promega's Reverse Transcription System and 15 units of AMV reverse transcriptase (Promega). We used both oligo(dT) and random primers (0.3 mg each) to ensure that entire mRNA strands were labeled. RNA was incubated at 70°C for 10 min to remove secondary structures. After all reagents were combined, the reaction was first incubated at room temperature for 10 min to allow extension of the random primers and then incubated at 45°C for 60 min. The reaction was terminated by incubating the sample at 95°C for 5 min, then incubating on ice for at least 5 min.

Nested PCR for UPs and EGFR

Primers and PCR conditions used for UP and EGFR PCR are outlined in Table I. First-round primers for UPs Ia, Ib and III as well as EGFR have been previously described.[21–23] We determined the suitability of specific sets of PCR primers based on satisfactory results seen in dilution experiments with the inclusion of negative controls as previously described.[24] All PCR experiments were performed in PCR buffer containing dNTPs, $MgCl_2$, oligonucleotide primers and Taq polymerase to a final volume of 25 μl and run on a Gene Amp PCR Thermocycler 9700 (Perkin-Elmer, Norwalk, CT). Every clinical sample was tested in 2 different RT-PCR assays. If both assays showed the correct band, the sample was classified as positive. If no band was detected in either of the 2 assays, the sample was considered negative. If discordance was observed between 2 reactions (a positive and negative result of the same sample), the sample was subjected to a third independent RT-PCR assay. If the third assay was positive, the sample was classified as positive. If the third assay was negative, the sample was classified as negative. We encountered discordance in 5 of 62 cases (4 were evaluated as negative after the third PCR and 1 was classified as positive).

Sequencing

Sequencing was performed on the RT-PCR products generated from the positive controls and 5 randomly selected positive patient samples for each marker. Bands representing RT-PCR products were excised from the agarose gel and purified using the Quantum Prep Freeze'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Richmond, CA). The sample was dried completely, combined with 25 μl of dH_2O and then rerun on agarose gel to quantify DNA for the Big Dye Terminator (Applied Biosystems, Foster City, CA). Ten nanograms of template per 100 bp of wanted sequence, used in conjunction with the Big Dye Terminator according to the manufacturer's instructions, underwent one round of PCR. Centri-Sep columns were used to remove excess terminators (Princeton Separations, Adelphia, NJ). The sample was dried completely, combined with 25 μl of Template Suppression Reagent (Applied Biosystems) and sequenced using the ABI Prism 310 Genetic Analyzer (Perkin-Elmer).

Statistical analysis

Patients with metastatic cancer were classified as disease-positive. Patients who had unresectable or recurrent pelvic disease were likewise classified because of their similar clinical courses as they almost invariably developed metastases during their limited survival.[25] Patients who underwent cystectomy or had no evidence of bladder cancer after definitive surgical treatment were classified as disease-negative. Defining the appropriate negative control for determining the sensitivity and specificity of the assay is a critical

TABLE I – OLIGONUCLEOTIDE PRIMERS AND PCR CONDITIONS USED FOR UP AND EGFR PCR

	Size (bp)	Round	Primer sequence 5′ → 3′ Sense/Antisense	Annealing temp (°C)	Cycles
UP1a	360	1st	ATGGCGTCTGCGGCAGCAGCG/ GAGCATGGTGTAGAAATACATG	53	30
		2nd	ACGTCCTACACCCACCGTGA/ ACCCCACGTGTAGCTGTCGAT	61	30
UP1b	363	1st	ATGGCCAAAGACAACTCAACT/ ATATTCAATTCTGCTCCAGTAG	53	30
		2nd	CAACGAGACTTTTTCACACCC/ CCCCCAGGCGTGTCGGTTCA	61	30
UPII	433	1st	CCATCCGGACCTTGCCCTTGATCCT/ GCCCAGTGCCAGGGCAATGATGAAG	66	30
		2nd	CCTGATTCTGCTGGCTCTGCTGT/ ACAGAGAGCAGCACCGTGATGAC	66	30
UPIII	392	1st	GCCAGTGTGACTTTCGCCACC/ TTGGAGCTTGCTGGAATAC	61	30
		2nd	ACAAACAGAGGGTGGGAGGACAG/ AGAAGGGCAGGGAGCCCAGG	61	30
EGFR	441	1st	CTTCTTGCAGCGATACAGCTC/ ATGCTCCAATAAATTCACTGC	58	30
		2nd	TAAACCAGTCCGTTCCCAAA/ GTCAGGGTTGTCCAGGCTAA	54	30

point in assessing the validity of the results. However, review of the scientific literature demonstrates that this issue has not been resolved. Some studies have used healthy donors and/or cystitis patients as negative controls.[21–23] We believe that if healthy donors and cystitis patients are considered the "no disease" cohort, then all patients with bladder cancer, regardless of disease stage, would be considered the "disease" cohort. The test is then designed for diagnosis. Alternatively, if RT-PCR is aimed at early detection of hematogenous disease and prognostication, then patients with metastatic bladder cancer should be considered the disease cohort and patients with localized bladder cancer with no evidence of disease posttreatment should compose the no disease cohort. Here, we followed the latter approach to determine the sensitivity and specificity of the assay. This same strategy has been recommended based on a meta-analysis of 1,799 melanoma patients analyzed in 23 studies between 1966 and 1999 to evaluate the utility of RT-PCR for tyrosinase mRNA as a marker for CTCs in cutaneous melanoma, which is a similar concept to using UP mRNA as a marker for CTCs in bladder cancer.[26]

Sensitivity and specificity were estimated by the corresponding sample proportions: *(i)* sensitivity was defined as the proportion of patients with a positive assay result among diseased patients and *(ii)* specificity was defined as the proportion of patients with a negative assay result among truly nondiseased patients at the end of the follow-up period. Exact confidence intervals for each measure were constructed using the Blythe-Still-Casella method.[27,28] Statistical analyses were carried out using StatXact version 4.0.1 (Cytel Software Corp., Cambridge, MA) and Stata version 7 (StataCorp, College Station, TX).

RESULTS

Sixty-two bladder cancer patients who presented to the Sidney Kimmel Center for Prostate and Urological Cancers at MSKCC between July 2000 and September 2001 were studied. Patient characteristics are summarized in Table II. Thirty-four (55%) patients had evidence of metastatic disease at the time of the assay. Patients with recurrent muscle-invasive disease and locally advanced disease represented a small proportion ($n = 6$, 10%) of this cohort.

The remaining 22 patients (35%) did not have evidence of disease at the time of the assay; however, 8 of these 22 patients relapsed during follow-up (median 15 months). Table III describes the characteristics of those 8 patients. UPII was positive in 6 (75%), UPIa in 4 (50%), and the combination of UPIa/UPII in 8/8 (100%) cases. All other markers tested individually and the combination of UPIb/UPIII were positive in 1–3 cases only.

Table IV demonstrates the sensitivity of all markers based on the proportion of patients with a positive assay among those with disease and the specificity of all markers based on the proportion of patients with a negative assay among those without disease at the end of follow-up. The sensitivity of the 5 markers tested individually ranged 21–54% and the specificity, 29–86%. While UPII and EGFR tested individually showed comparable sensitivity (52% and 54%, respectively), which was higher than that of all other markers, the specificity of UPII was considerably better than that of EGFR (64% *vs.* 29%, respectively). Figure 1 shows representative cases studied for UPII. Both UPIb and UPIII demonstrated a very similar pattern when tested individually. They showed relatively high specificity (86% and 79%, respectively) and relatively modest sensitivity (21% and 23%, respectively). Figure 2 shows cases studied for UPIa, UPIb and EGFR.

The strategy of testing specific paired combinations was based on our previously published data showing that UPIa and UPIb form heterodimers with UPII and UPIII, respectively.[10,11] The assay for the UPIa/UPII pair provided the highest sensitivity (75%) for detecting the presence of disease in this cohort. However, the specificity of the UPIa/UPII assay was not as high as that of some of the markers tested individually (*e.g.,* UPIb and UPIII). The combination of UPIb/UPIII was the most specific (79%); however, its limited sensitivity (31%) greatly limits its usefulness. Detection of EGFR-positive CTCs was inferior (sensitivity 54%, specificity 29%) to that for the UPIa/UPII combination (Table IV). We also explored the combinations of EGFR/UPIa/UPII and EGFR/UPIb/UPIII. Our analyses revealed that these combinations did not improve the sensitivity/specificity profile achieved by UPIa/UPII (data not shown).

Table V illustrates the positive detection rate of CTCs at specific sites of metastases. Forty-two (34 at the time of assay and 8 on follow-up) patients had evidence of disease at 60 sites grouped in 6 different categories (bone, brain, liver, lung, lymph node/soft tissues and others). Interestingly, patients with lung metastases ($n = 7$) showed the highest rate of positive CTC detection as EGFR-positive CTCs were detected in all 7 cases (100%) and UPIa/UPII-positive CTC were observed in 6 (86%). Comparable percentages were observed for other sites (Table V). Both the combination of UPIa/UPII and EGFR provided similarly high positive rates in patients with metastases compared to other markers.

TABLE II – CHARACTERISTICS OF 62 BLADDER CANCER PATIENTS TESTED FOR CIRCULATING TUMOR CELLS EXPRESSING UROTHELIAL AND EPITHELIAL MARKERS

Patient #	62	
Age	Range 42–88 (mean = 64 years)	
Gender distribution	14 Female (23%)	
	48 Male (77%)	
Disease status at time of CTC assay	No evidence of disease:	22 (35%)
	Presence of disease:	40 (65%)
	Metastatic disease:	34 (55%)
	Recurrent, muscle invasive:	1 (2%)
	Unresectable:	5 (8%)
Disease status at the end of the study follow up period	No evidence of disease:	14 (23%)
	Presence of disease:	48 (77%)
	Metastatic disease:	42 (67%)
	Recurrent, muscle invasive:	1 (2%)
	Unresectable:	5 (8%)
Site of disease:	Bone: 8	
	Brain: 1	
	Liver: 7	
	Lung: 7	
	Lymph node/soft tissue: 29	
	Other: 8[1]	
Median follow up	15 months (range 5–23)	

[1]Adrenal (1), mesentery (3), local bladder recurrence (4).

DISCUSSION

We report on the detection of 4 forms of UP and EGFR in the blood of a large number of bladder cancer patients over an extended period of follow-up. Our study demonstrates several points. First, the use of appropriate combinations of urothelium-specific markers (UPIa/UPII) is superior to that of a single urothelial or epithelial marker at detecting CTCs of bladder cancer patients. Second, there is considerable heterogeneity in the expression of urothelial and epithelial markers in individual bladder cancer patients. Third, our observation that all bladder cancer patients who relapsed were positive for UP1a/UPII despite showing no evidence of disease at the time of the initial assay suggests that our assay can be helpful in predicting disease recurrence in bladder cancer patients.

The UP-based assay for the detection of CTCs in bladder cancer has been tested in a number of studies since UPs were cloned a decade ago.[17,21,22,29] Yuasa *et al.,* who reported on a small cohort of 12 bladder cancer patients, analyzed UPIa and UPII in one publication[22] and UPIb and UPIII in another.[21] However, the small number of patients makes it impossible to draw clinically meaningful conclusions. Our group has previously reported UPII transcript detection rates of 30% in the blood of metastatic bladder cancer patients and 0% in individ-

TABLE III – CLINICAL AND PATHOLOGICAL FEATURES OF PATIENTS WHO SHOWED POSITIVE CTC IN THE ABSENCE OF CLINICAL DISEASE AT THE TIME OF THE INITIAL ASSAY BUT RELAPSED DURING FOLLOW UP

Patient no.	TNM	Grade	Surgical treatment	Date of initial assay	Date of recurrence	Months to recurrence
1	T3bN2M0	High	Cystectomy BPLND	07/25/00	11/07/01	16.0
2	T3bN0M0 T3N0M0	High	Cystectomy BPLND Penectomy	08/01/00	09/12/00	1.0
3	T3N0M0 TisN0M0	High	Lnephroureterectomy TURBT	11/28/00	03/30/01	4.0
4	TaN0M0	Low	TURBT	11/28/00	09/18/02	22.0
5	T3bN2M0	High	Cystectomy BPLND	12/05/00	02/27/01	2.0
6	T1N0M0	High	TURBT	12/12/00	06/19/01	6.0
7	T4N2M0	High	Cystectomy BPLND	01/16/01	06/22/01	5.0
8	T3N2M0	High	Cystectomy BPLND	03/06/01	07/30/01	4.0

TABLE IV – THE SENSITIVITY AND SPECIFICITY OF CIRCULATING TUMOR CELLS EXPRESSING UROTHELIAL AND EPITHELIAL MARKERS IN 62 BLADDER CANCER PATIENTS

Assay	Sensitivity[1] (95% CI)	Specificity[1] (95% CI)
UPIa	44% (29%, 59%)	64% (37%, 85%)
UPII	52% (37%, 66%)	64% (37%, 85%)
UPIa/UPII[2]	75% (60%, 86%)	50% (23%, 77%)
UP1b	21% (10%, 34%)	86% (58%, 97%)
UPIb/UPIII[2]	31% (19%, 45%)	79% (50%, 94%)
UPIII	23% (13%, 37%)	79% (50%, 94%)
EGFR	54% (39%, 69%)	29% (10%, 58%)

[1]Sensitivity and specificity of the assay were calculated based on disease status at the end of follow up period.–[2]For the dimers UPIa/UPII and UPIb/UPIII, the following outcomes were considered to represent a positive assay: positive/positive, positive/negative, negative/positive.

uals with nonmetastatic tumors. Li *et al.*[17] employed RT-PCR for only UPII; the use of a single marker might explain the limited detection rate in patients with clinical evidence of metastases. Lu *et al.*[29] utilized a nested RT-PCR to increase the sensitivity of the PCR assay. They also focused solely on UPII and reported that its positive detection rate increased with stage in a statistically significant fashion.

To better address the adequacy of the sample size, we compiled a relatively large, well-characterized cohort ($n = 62$) of bladder cancer patients with clinical follow-up (median 15 months, range 5–23). The relatively short period of follow-up for some cases reflects the advanced disease status in a significant proportion of our study cohort. In addition, we expanded our study to include all 4 major forms of UP. We also included EGFR as a marker because it is associated with urothelial basal cells and is known to be overexpressed in the less differentiated urothelial tumors. Studying EGFR is also important because of the availability of several anti-EGFR compounds (both MAbs and small peptides) in clinical trials.[30–33] An assay that can detect EGFR-positive CTCs may help identify appropriate candidates for trials using anti-EGFR strategies. Gazzaniga *et al.*[23] reported that EGFR might have a better specificity profile compared to UPII for detecting CTCs in bladder cancer patients. The investigators detected EGFR mRNA in the blood of patients with bladder carcinoma of various stages. Interestingly, they also reported that most of the nonmetastatic patients whose blood was EGFR-positive at the time of diagnosis relapsed on follow-up. They compared EGFR with one form of UP (UPII). Our data indicate, however, that the combination of UPIa/UPII may be superior to EGFR in sensitivity and in predicting disease relapse in the setting of bladder cancer.

Our analysis of the detection of CTCs in metastatic disease revealed several interesting points. First, patients with lung metastases had the highest rate of positive CTCs. Nevertheless, several patients with lung cancer metastases also had evidence of metastases at other sites. This precludes us from drawing conclusions regarding an association between CTCs and the specific clinical behavior of bladder cancer. Second, all 8 patients who did not have evidence of clinically detectable disease at the time of the initial CTC assay but later relapsed were positive for UPIa/UPII. This implies that detection of this combination of UP transcripts in the blood of a subset of patients may be useful in predicting disease recurrence.

There are several limitations in our study. First, the assay was done at only one time point; since tumor cells may shed intermittently into the circulation, this might lead to one-time false-negative results. Second, there was a relatively small proportion of patients with no evidence of disease ($n = 22$) at the time of the study. This prevented us from determining the predictive value of

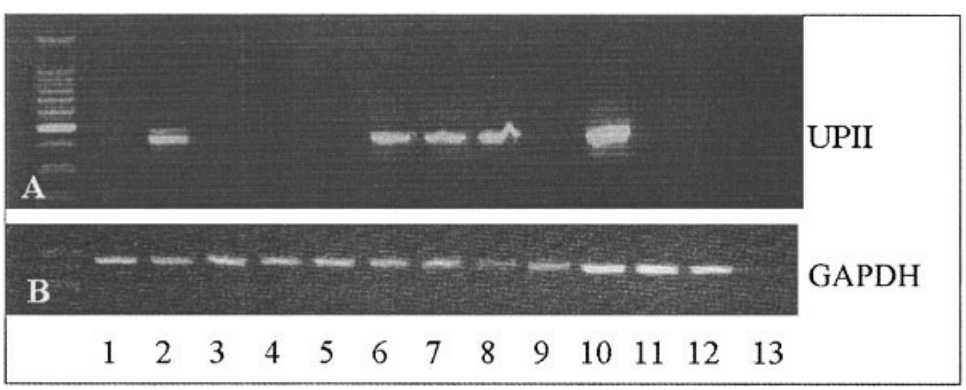

FIGURE 1 – *(a)* Nested RT-PCR for UPII. Lanes 1–9, patient samples; lane 10, RT4 TCC (UP-positive); lane 11, fibroblast 3T3 (UPII-negative); lane 12, healthy volunteer sample (negative control); lane 13, H_2O (PCR negative control). *(b)* RT-PCR for GAPDH as control for RNA integrity.

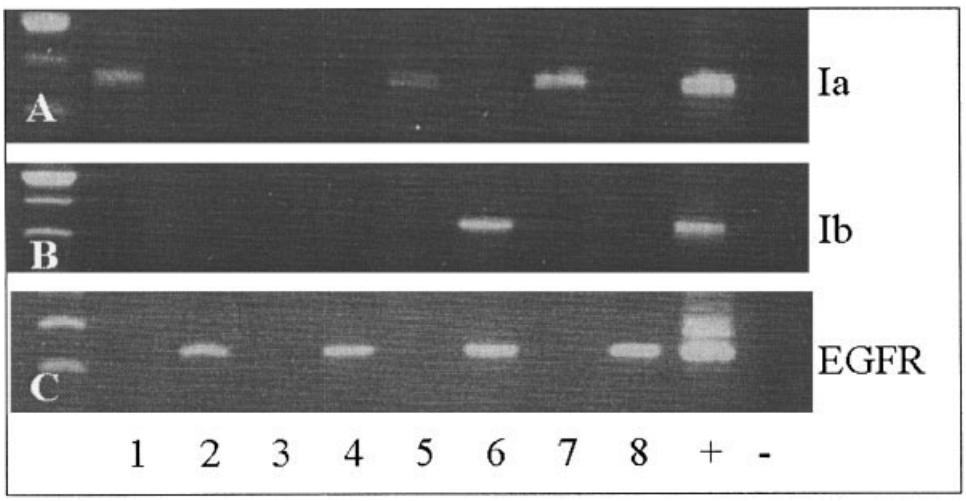

FIGURE 2 – *(a)* RT-PCR for UPIa. *(b)* RT-PCR for UPIb. *(c)* RT-PCR for EGFR. All panels: lanes 1–8, patient samples; lane 9, positive control, RT4 TCC cell line; lane 10, negative control, normal volunteer blood.

TABLE V – DETECTION RATES OF CTC AT SPECIFIC SITES OF METASTASES

Marker (+)	Bone (n = 8)	Brain (n = 1)	Liver (n = 7)	Lung (n = 7)	LN/ST (n = 29)	Other (n = 8)
UP Ia	4/8 (50%)	0	2/7 (29%)	4/7 (57%)	10/29 (34%)	3/8 (37.5%)
UP II	3/8 (37.5%)	0	3/7 (43%)	6/7 (86%)	16/29 (55%)	3/8 (37.5%)
UP II/Ia	5/8 (62.5%)	0	4/7 (57%)	6/7 (86%)	20/29 (69%)	5/8 (62.5%)
UP Ib	1/8 (12.5%)	0	2/7 (29%)	2/7 (29%)	8/29 (28%)	3/8 (37.5%)
UP III	2/8 (25%)	0	3/7 (43%)	2/7 (29%)	8/29 (28%)	1/8 (12.5%)
UP III/Ib	2/8 (25%)	0	3/7 (43%)	3/7 (43%)	11/29 (38%)	3/8 (37.5%)
EGFR	6/8 (75%)	0	3/7 (43%)	7/7 (100%)	18/29 (62%)	3/8 (37.5%)

the markers studied. Third, a certain percentage of patients with localized disease will experience recurrence with time; thus, these patients can be expected to harbor CTCs. To address these limitations, we have begun a prospective study of a larger number of bladder cancer patients. We will perform the CTC detection at several different time points to account for the possibility of intermittent shedding into the circulation. The true "no hematogenous disease" cohort will be comprised of patients with low-grade bladder cancer who have a 95% chance of surviving 5 or 10 years without evidence of recurrence. In addition, we will include patients with muscle-invasive disease to evaluate the true positive predictive value of the assay.

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