Molecular Staging of Bladder Cancer with RT-PCR Assay for CK20 in Peripheral Blood, Bone Marrow and Lymph Nodes: Comparison with Standard Histological Staging

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Abstract. *Background: The aim of this study was to analyze whether the CK20 reverse transcriptase polymerase chain reaction (RT-PCR) is suitable for detecting circulating tumor cells and residual tumor cells in lymph nodes, in patients with muscle invasive transitional cell carcinoma (TCC) of the bladder, and to compare these results with standard histological staging. Patients and Methods: The nested RT-PCR assay was used to analyze the CK20 transcript in the peripheral blood, bone marrow, lymph nodes, the tumor and normal biopsies of bladder from 57 patients with invasive TCC of the bladder, who underwent radical cystectomy, and from 9 patients with non-invasive TCC. Results: Lymph node pathological status was positive in 24 out of the 57 patients studied and all of them except 1 showed expression of CK20, with a correlation between histological technique and RT-PCR of 95.8%. A statistically significant correlation of lymph node CK20 RT-PCR with the standard risk factor of pathological stage (p=0.04) was observed. Blood and bone marrow CK20 RT-PCR showed no correlation with pathological stage. Conclusion: Lymph node CK 20 RT-PCR correlates with pathological stage in bladder cancer. The CK20 RT-PCR assay appears to be a highly sensitive and specific method for detecting circulating tumor cells and residual disease in lymph nodes in patients with invasive bladder cancer. Further evaluation of the significance of CK20 as a molecular marker for staging and follow-up in these patients is necessary.*

Urothelial cancer is the second most common urological malignancy and, despite advances in the diagnosis and therapy of bladder cancer, its overall prognosis has not markedly improved. The 5-year survival rate of patients with muscle-invasive disease, despite radical cystectomy, ranges between 39-53% (1). Half of our patients with localized bladder cancer develop metastases in the first 2 years of follow-up and none of them can be identified in the pre-operative assessment with standard clinical staging methods like scintigraphy or computed tomography (CT) scan. Systemic progression could be due to isolated disseminated bladder cancer cells undetectable at the initial assessment (2).

CK20 is an epithelial cell intermediate filament, whose expression is almost entirely confined to the gastric and intestinal epithelium, urothelium and Merkel cells. The expression spectrum of CK20 in carcinomas resembles that seen in the corresponding normal epithelia of origin (3). CK20 has been proposed as a new tool for the detection of circulating tumor cells in the peripheral blood and bone marrow of bladder cancer patients (2, 4, 5) For patients with no evidence of systemic disease when the primary tumor is resected, traditional staging parameters, such as pathological stage and lymph node involvement, are required for assessment of the risk of disease recurrence. These data provide the evidence for the decision to give systemic adjuvant treatment to prevent metastatic relapse (6). The identification of occult metastases in patients with localized bladder cancer could have an impact on the optimal treatment and prognosis of these patients.

Several studies have analyzed circulating tumor cells in bladder cancer (4, 5, 7-13), while another study analyzed residual disease in lymph nodes in patients with urothelial carcinoma of the bladder (14). Some authors concluded that the molecular staging of bladder cancer has prognostic value. To our knowledge, there are no reports of the simultaneous study of hematogenous and lymphatic dissemination in bladder cancer by molecular techniques.

In the present study, the detection of bladder cancer cells in pelvic lymph nodes was investigated by CK20 reverse

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transcriptase polymerase chain reaction (RT-PCR) *versus* standard histology and these results were compared to hematogenous detection by CK20 RT-PCR in peripheral blood and bone marrow samples. With the ongoing long-term clinical follow-up of this patient cohort, whether RT-PCR can be helpful to determine which type of adjuvant therapy should be chosen for patients with invasive bladder cancer, submitted to radical cystectomy, will be evaluated.

Patients and Methods

Patients. A total of 66 patients with diagnoses of transitional cell carcinoma (TCC) of the bladder were included in this study (25 patients treated at the Department of Urology of the Hospital Clinic, Barcelona, and 41 who consulted the Department of Urology at Fundació Puigvert, Barcelona, Spain). Fifty-seven of these patients underwent radical cystectomy (53 because of muscle-invasive bladder cancer and the remaining 4 because of T1G3 TCC with no response to BCG therapy) (Table Ia). The remaining 9 were subjected to transurethral resection because of superficial TCC (Table Ib). The grade and stage of the tumors were determined according to WHO criteria (15) and TNM classification (16), respectively.

Control samples. To test the specificity of the CK20 RT-PCR assay, 22 blood samples of healthy donors, 5 lymph node samples and 5 bone marrow samples from patients with prostate carcinoma were included as negative controls. Blood samples from 4 metastatic patients were included as positive controls.

Ethical issues. The Bioethics Committee of both hospitals approved this project and written informed consent was obtained from all those partaking in the study.

Blood and bone marrow collection. Peripheral blood and bone marrow samples were collected from the patients before surgery and processed immediately after extraction. The bone marrow samples were collected only from the first 25 patients with invasive TCC included in the study. To avoid contamination by Merkel cells, 2 samples of peripheral blood were obtained by venipuncture using EDTA tubes, and only the second one containing 10 ml was processed to obtain the RNA.

Tumor, lymph nodes and normal bladder specimens. Bladder and lymph node specimens were sent to the Department of Pathology immediately after resection. The obtained biopsy specimens of bladder tumors, normal bladder tissue and lymph nodes were frozen in liquid nitrogen and stored at –80˚C until processing. One-half of each lymph node was used for standard histopathology and the rest for the detection of CK20 expression.

RNA extraction. Mononuclear cells from blood and bone marrow samples were isolated from 10 ml of both types of samples by centrifugation techniques (Ficoll-Paque, Pharmacia, Uppsala, Sweden). The total RNA was extracted from mononuclear cells by using a commercially available extraction kit (TriPure isolation reagent, Roche, Nutley, NJ, USA), according to the manufacturer's protocol. The total RNA from approximate 50-100 mg of frozen biopsy specimens of bladder tumor, normal bladder tissue and lymph node was extracted, after mechanical homogenization, with the same isolation reagent.

The amount and purity of the extracted RNA was determined from absorption at 260 nm and 280 nm (Pharmacia). The integrity of the RNA was determined by visualizing the ribosomic RNA in 1% agarose gel electrophoresis.

RT-PCR. The cDNA was synthesized with a reverse transcription kit (SuperScript II; Invitrogen, Carlsbad, CA, USA), using 6-10 μg of the total RNA and the CK20 558rev primer (5) in a total volume of 20 μl. The *CK20* gene was amplified in subsequent PCRs. The reaction components for each PCR were 1X buffer, 200 μM dNTP, 1.5 mM $MgCl_2$ and 0.5 U of Taq Polymerase (Ecogen, Spain) in a total reaction volume of 25 μl. The first PCR was performed with 2 μl of cDNA and 1.6 μM each of the 1F and 558R primers (5). Forty cycles of amplification were performed with a denaturation temperature of 94˚C for 30 sec, an annealing temperature of 60˚C for 30 sec and an extension temperature of 72˚C for 30 sec. Subsequently, 2 μl of the first PCR were subjected to a nested PCR. The nested PCR reaction was performed in a total volume of 25 μl with 1.6 μM each of the 139F and 429R primers (5). Thirty-five cycles of amplification were performed with a denaturation temperature of 94˚C for 30 sec, an annealing temperature of 72˚C for 30 sec and an extension temperature of 72˚C for 30 sec. Each RNA sample isolated from mononuclear cells of blood and lymph nodes was subjected to the nested PCR twice. A sample was considered positive if 1 out of 2 reactions gave a positive result for CK20 amplification. Negative control samples were run in parallel with each experiment in which cDNA and the product of the first PCR were replaced with water. The nested PCR products were analyzed by electrophoresis on 2% agarose gels (Figure 1).

Furthermore, about 50% of the samples that did not amplify CK20 mRNA were subjected to glyceraldehyde-3-phospate dehydrogenase (GAPDH) RT-PCR to check the integrity of the isolated RNA (17).

To confirm the specificity of the PCR for CK20, some randomly-chosen PCR products were gel-excised according to the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) protocol. These PCR fragments were subsequently sequenced using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol.

Statistical analysis. The χ^2 test or Fisher's exact test were used to examine the correlation between the CK20 expression in the different samples and the rest of the parameters tested. The SPSS software, version 10.0 (SPSS Corp., Chicago, IL, USA), was employed and the test was evaluated by applying a 0.05 significance level.

Results

Patients' characteristics and tumor stages. The characteristics of the 66 tumors, including clinical / pathological stages, are shown in Table Ib. The patient cohort consisted of 62 men and 4 women, with a median age of 65 years (range 44-81). Twenty-five out of the 53 (47%) patients with muscle-invasive bladder cancer, who underwent cystectomy, had a tumor pathological stage higher than the clinical stage. At the final pathological analysis, 17 patients showed pT2G3 TCC, 15 pT3G3, 14 pT4G3, 5 showed residual superficial TCC (Ta-T1), 3 pT0 and 3 patients out of 4 who had been submitted to radical cystectomy for superficial bladder cancer

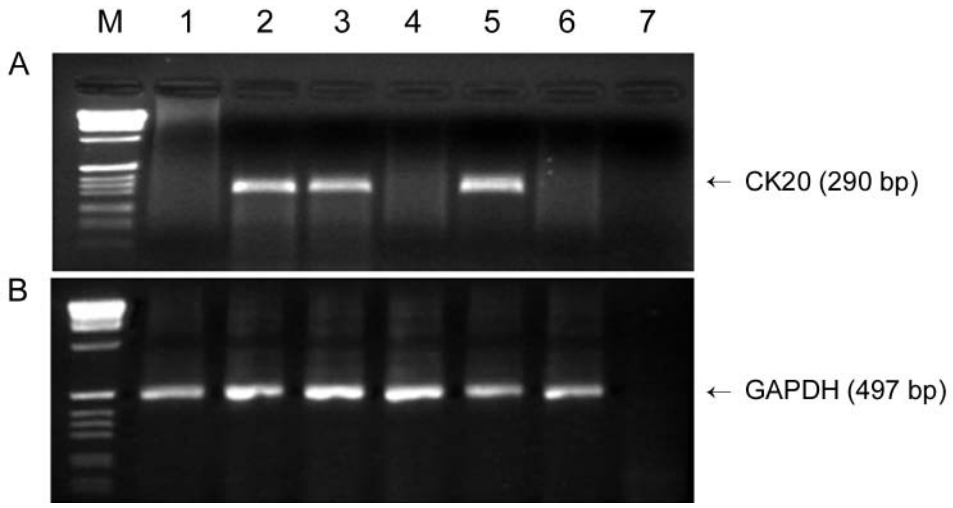

Figure 1. *A) Detection of mRNA for the CK20 gene by RT-PCR in a representative patient with TCC of the bladder. The PCR products for CK20 (290 bp) were positive in lanes 2, 3 and 5. B) RT-PCR analysis of the same set of RNA samples for glyceraldehyde-3-phosphate dehydrogenase, as an internal control for the integrity of the RNA (497 bp). Normal (1) and tumoral (2) biopsy of bladder, right (3) and left (4) lymph nodes, bone marrow (5) and (7) negative control of water. M= molecular size marker.*

with no response to BCG showed non-invasive bladder cancer. In the group of patients with non-invasive bladder cancer that underwent transurethral resection (TUR), 1 showed TaG1 TCC, 3 TaG2, 1 Ta G3, 2 T1G2, 1 T1G3 and 1 T1G3, with associated Cisplatin and BCG therapy failure, thus absolute medical contraindication for radical surgery.

The pathological status of the lymph nodes and matched CK20 RT-PCR assay were available in 54 patients out of the 57 submitted to radical cystectomy. From this group of patients, 24 (44%) showed lymph node tumoral involvement in the pathological analysis.

CK20 expression in biopsy specimens of bladder tumors and normal bladder tissue. The CK20 expression was studied in 60 biopsies of bladder tumors and 63 macroscopic normal bladder tissues by the RT-PCR technique. The CK20 transcripts were detected in 92% (58/63) of the normal biopsies studied and 93.3% (56/60) of the tumor biopsies studied.

CK20 expression in control samples. CK20 expression could neither be detected in the mononuclear blood cells from the 22 negative control samples, nor in the 5 bone marrow aspirates from patients suffering from prostate cancer. CK20 transcripts were also undetectable in 5 lymph nodes of these latter 5 patients. On the other hand, CK20 expression was detected in 3 out of 4 blood samples from metastatic bladder cancer patients.

CK20 expression in blood and bone marrow samples from patients with TCC. The CK20 expression was studied in 57 blood samples and 25 bone marrow samples from patients with TCC who underwent radical cystectomy. CK20 expression was detected in 33.3% (19/57) of the blood samples and 16% (4/25) of the bone marrow samples studied (Table Ia).

CK20 transcripts were not detected in 8 out of 9 blood samples (88.9%) from patients with superficial TCC of the bladder. Only 1 blood sample, corresponding to a stage T1G3+Cis with non-response to BCG therapy and absolute contraindication for radical cystectomy, resulted positive for the expression of CK20.

CK20 expression in lymph nodes from patients with TCC. The CK20 expression was evaluated in 99 lymph nodes (51 right nodes and 48 left nodes) from the 54 patients with invasive TCC who underwent cystectomy. Forty patients out of the 54 (74%) showed CK20 expression upon lymph node analysis, while only 24 out of the 54 patients (44.4%) showed lymph node tumor involvement on pathological analysis. All patients but 1 (95.8%) with node-positive disease on pathological analysis showed CK20 expression with the RT-PCR assay. Among the 30 remaining patients with no histological lymph node metastases, RT-PCR analysis showed micrometastases in 14 (46.6%).

Correlation between CK20 expression and tumor stage. A statistically significant correlation was observed between lymph node histopathology and tumor stage ($p=0.001$) since 5 out of the 25 (20%) patients with lymph node involvement on pathological analysis showed T2 stage, while 20 (80%) showed T3-T4 TCC. A significant correlation could also be observed between lymph node CK20 expression and tumor stage, since 16 out of the 40 (40%) patients with CK20

Table I.
a *Summary of CK20 expression in different samples studied.*

	CK20 expression		Total	% of positive cases
	Positive	Negative		
Blood	19	38	57	33.3
Bone marrow	4	21	25	16
Normal biopsy	50	4	54	92.6
Tumoral biopsy	48	4	52	92.3
Righ lymph nodes	33	18	51	64.7
Left lymph nodes	27	21	48	56.3
Right+left lymph nodes	40	14	54	74.1

positivity in the nodal sample showed T2 TCC, while 24 (60%) showed T3-T4 TCC ($p=0.043$).

Seven out of the 19 (36%) patients with CK20 positivity in the peripheral blood had T2 TCC and 12 (63%) showed T3-T4 TCC; no statistically significant association was found between CK20 expression in the blood samples and the stage of the disease ($p=0.26$). CK20 expression in the bone marrow could be demonstrated in 4 out of 25 (16%) patients and all of them showed T3-T4 TCC on pathological analysis. Again, no statistical correlation was found between CK20 expression in the bone marrow and the stage of the disease ($p=0.26$).

b *Clinicopathological profiles and detection of CK20 mRNA-positive cells in peripheral blood, bone marrow and lymph nodes of patients with bladder cancer. (B=peripheral blood, NB=normal bladder biopsy, TB=tumor biopsy, RLN=right lymph node, LLN=left lymph node, BM=bone marrow).*

Patient number	Clinical stage	Lymphadenectomy		Pathological stage	RT-PCR CK20					
		Right	Left		B	NB	TB	RLN	LLN	BM
Patients who underwent cystectomy										
1	T2G3N0M0+cis	+	-	T3bG3N1M0	+	+	+	+	ND	-
2	T1G3N0M0+cis	-	-	T1G3 N0M0+cis	-	+	+	-	-	-
3	T2G3N0M0	-	-	T4G3N0M0	-	+	+	-	-	-
4	T2G3N0M0	-	-	T2G3N0M0	-	+	+	+	+	-
5	T3G3N0M0	+	-	T4bG3N1M0	-	+	+	+	+	+
6	T2G3N0M0	-	-	T2G3N0M0	-	+	+	-	-	-
7	T2G3N0M0	+	+	T4bG3N2Mx	+	+	+	+	+	+
8	T2G3N0M0	-	-	T2G3N0M0	+	+	+	+	+	-
9	T3G3NOM1	+		T3G3N1M1	+	-	+	ND	+	-
10	T2G3N0M0	-	-	T2G3N0M0	-	+	+	-	-	-
11	T3G3N1M0	-	+	T4G3N1M0	-	+	+	-	+	-
12	T3G3N0M0	+	-	T4G3N1M0	+	-	+	+	-	-
13	T2G3N0M0	+		T3bG3N2M0	+	+	+	+	+	-
14	T3G3N0M0	+		T3bG3N1M0	-	+	+	+	-	-
15	T2G3N0M0	-	-	T2G3N0M0	-	-	+	-	-	-
16	T2G3N0M0	+		T3bN1M0	-	+	+	+		-
17	T2G3N0M0	+	+	T3G3N2M0	+	+	+	+	+	+
18	T2G3N0M0	+	-	T3G3N2M0	-	+	+	+	+	-
19	T2G3N0M0	-	-	T2G3N0M0	-	+	+	-	-	-
20	T1G3N0M0+cis	-	-	T3G3N0M0	-	+	+	+	+	-
21	T2G3N1M0	-	-	T4G3N0M0	-	+	+	ND	ND	-
22	T2G3N0M0	-	-	T4G3N1M0+Cis	+	+	+	-	+	+
23	T2G3N0M0	-	-	T2bG3N0M0+Cis	+	+	+	-	+	-
24	T2G3N0M0+cis	+		T2G3N1M0+Cis	-	ND	ND	+	ND	-
25	T2G3N1M0+cis	-	+	T3G3N1M0+Cis	+	+	+	+	ND	-
26	T2bG3N0M0	-	-	T0N0M0	+	-	ND	-	-	ND
27	T2G3N0M0	+	+	pT4aG3N2M0	+	+	+	+	+	ND
28	T3aG3N0M0	-	-	pT3bG3N0M0	+	+	+	+	+	ND
29	T2G3N1Mx	-	-	pT1G3N0Mx	+	+	+	+	+	ND
30	T2bG3N0M0	-	-	pT2aG3N0M0+Cis	-	+	+	+	+	ND
31	T2G3N0M0	-	-	pT2aG3N0M0	-	+	+	+	-	ND
32	T2G3N0M0	-	-	pT2aG3N0M0	+	+	-	+	-	ND
33	T2G3N0M0+cis	-	-	pT2bG3N0M0	-	+	+	-	-	ND
34	T2aG3N1M0	-	+	pT2bG3N2M0	+	+	+	ND	+	ND
35	T3G3N0M0	+	-	pT4aG3N2M1	-	ND	ND	+	+	ND
36	T2aG3N0M0	ND	+	pT3bG3N2M0	+	+	+	ND	-	ND

Table I b continued

Table I b continued

Patient number	Clinical stage	Lymphadenectomy		Pathological stage	RT-PCR CK20					
		Right	Left		B	NB	TB	RLN	LLN	BM
37	T2G3N1M0	+	+	pT4G3N2M0	-	+	-	+	ND	ND
38	T2bG3N0M0	+	+	pT4aG3N2M0	-	+	ND	+	-	ND
39	T2bG3N0M0+cis	-	-	pT3aG3N0M0	-	+	+	-	-	ND
40	T4G3N0M0	-	-	pT4G3N0M0	-	+	-	-	-	ND
41	T2G3N0M0	-	-	pT0N0M0	-	+	ND	-	-	ND
42	T2G3N0M0	ND	ND	pT3bNxM0	+	+	+	ND	ND	ND
43	T2G3NxM0	+	+	pT1bG3N2M0	-	+	+	+	+	ND
44	T2bG3N0M0+cis	+	-	pT2aG3N1M0+Cis	+	+	+	+	ND	ND
45	T2G3N0M0	+	+	pT4aG3N2M0	-	+	+	+	+	ND
46	T2G3N0M0+cis	-	-	pT3bG3N0M0	-	+	+	-	+	ND
47	T2G3N0M0	-	-	pT2bG3N0M0	-	+	+	+	+	ND
48	T2G3N0M0	ND	ND	pT2bG3NxM0	-	+	+	ND	ND	ND
49	T2aG2N0M0	-	-	pT3aG3N0M0	-	ND	+	+	+	ND
50	T2aG2N0M0	-	-	pTaG2N0M0	-	+	+	+	+	ND
51	T2aG3N0M0+cis	-	-	pT1G3N0M0+Cis	-	+	+	+	-	ND
52	T2G3N0M0	-	-	pT2banaplasicN0M0	-	+	+	+	-	ND
53	T2G2N0M0	+	-	pT4aG3N2M0	-	+	+	+	+	ND
54	T3aG3N0M0	-	-	pT0N0M0	-	+	+	-	+	ND
55	T1G3N0M0+cis	-	-	pT1G3N0M0+Cis	-	+	+	-	-	ND
56	T1bG3N0M0+T1G3 in neck	-	-	pTaG2N0M0	-	+	+	-	-	ND
57	T2G3N0M0+cis	+	-	pT1G3N1M0	-	+	+	+	+	ND
Patients with superficial TCC of the bladder										
58	TaG2	ND	ND	ND	-	-	+	ND	ND	ND
59	TaG2	ND	ND	ND	-	+	ND	ND	ND	ND
60	T1G2	ND	ND	ND	-	+	+	ND	ND	ND
61	T1G3	ND	ND	ND	-	+	+	ND	ND	ND
62	TaG2	ND	ND	ND	-	+	-	ND	ND	ND
63	T1G3+cis	ND	ND	ND	+	+	+	ND	ND	ND
64	TaG3	ND	ND	ND	-	+	+	ND	ND	ND
65	TaG1	ND	ND	ND	-	+	+	ND	ND	ND
66	T1G2	ND	ND	ND	-	+	+	ND	ND	ND

Correlation between CK20 expression in blood, bone marrow and lymph nodes. The results of CK20 RT-PCR in the blood samples and lymph nodes were matched in 28 (51.9%) cases (16 positive and 12 negative for both types of samples). Twenty-four patients (44.4%) showed expression of CK20 mRNA in the lymph nodes and undetectable expression in the blood, while only 2 patients (3.7%) showed expression in the blood and undetectable expression in the lymph nodes. No statistically significant association was found between the expression of CK20 in both types of samples ($p=0.07$), while it is important to point out that 96.3% of the patients who showed CK20 expression in the peripheral blood also showed simultaneous CK20 expression in the lymph nodes.

Matching results of CK20 RT-PCR in bone marrow samples and lymph nodes were found in 10 (41.7%) patients (4 positive and 6 negative for both types of samples). Fourteen (58.3%) samples resulted positive for the expression of CK20 in the lymph nodes and negative in the bone marrow. No positive samples for the expression of CK20 in the bone marrow and no negative samples in the lymph nodes were found. In addition, no statistically significant relationship was found between these 2 parameters ($p=0.539$).

PCR product sequencing in the CK20 RT-PCR assay. To confirm the specificity of PCR for CK20, the PCR products of 5 samples positive for CK20 expression were sequenced. Sequencing of the final PCR products revealed that the amplified products were consistently identical to the CK20 cDNA sequence.

Discussion

Previous studies have focused on circulating tumor cells and micrometastases in bladder cancer (Table II). None of

Table II. *Comparative analysis of molecular studies on the detection of circulating tumor cells in peripheral blood, bone marrow and micrometastasis in lymph nodes of patients with bladder cancer.*

Authors	Year	No. of Patients	Stage of TCC studied	Marker	Technique	Samples			Results
						Blood	Nodes	Bone marrow	
Yuasa *et al.* (7)	1999	12	5 superficial TCC. 4 T2-3b. 3 N+M1	UPIaII	RT-PCR	■			All patients with node involvement or M1 show UPII expression. None of the superficial or non-metastatic. No expression in controls.
Fujii *et al.* (4)	1999	40	Upper urinary tract, urethra and bladder tumours. 13 superficial. 21 T2-4 N0M0. 6N+ or M1	CK20	RT-PCR	■			22.5% expression in TCC. No expression in superficial TCC. 19% T2-4 were CK20+. 83% of N+ or M1 were CK20+. No expression in controls.
Li *et al.* (9)	1999	60	Invasive, non-invasive and metastatic	UPII	RT-PCR	■			30% expression in metastatic TCC. No expression in non-metastatic. No expression in controls.
Güdeman *et al.* (5)	2000	49	22 superficial TCC. 19 T2-3. 8 M1	CK20	RT-PCR	■			No expression in Ta TCC. CK20+ in: 18% T1, 23% T2, 33% T3 and 63% of T4 or M1. No expression in controls.
Lu *et al.* (8)	2000	56	14T2-4N0M0 29 Ta-1N0M0 5N+. 8M1.	UPII	RT-PCR	■			UPII + in: 10% superficial TCC, 28% of T2-4, 40% of N+ and 75% of M1 TCC.No expression in controls. Good correlation with stage.
Seraj *et al.* (14)	2001	27	T1- T4a	UPII	RT-PCR		■		42% UPII+ perivesical tissue in organ-confined TCC. 25% of negative nodes in pathological analysis were + for UPII
Gazzaniga *et al.* (10)	2001	27	6 Tis-T1N0M0 10 T2-4N0M0 3N1M0 8M1	UPII CK20 CK19 EGFR	RT-PCR	■			70% TCC were EGFR+: 90% of invasive TCC, 100% of N1 and 87% of M1. CK20 in 15% TCC. UPII in 37%. CK19+ in 20% of controls.
Soria *et al.* (11)	2002	30	Muscle-invasive and metastatic	Telomerase	Inmuno-magentic beads labeled with specific antibody	■			90% of patients expressed telomerase. None of the controls telomerase+. No correlation with stage and grade
Retz *et al.* (2)	2001	29	11 superficial. 18 T2a-4aM0	CK20	RT-PCR	■		■	35% CK20+ in bone marrow (all T2 but 1 T1+Cis). 2 blood samples CK20+ preoperatively. No expression of CK20 in controls.
Hofmann *et al.* (12)	2003	128	Ta- T4. Bladder and upper urinary tract tumors	CK18	IHQ			■	29.7% TCC were CK18+. Statistical correlation with stage, grade, lymph node involvement and progression. No expression in controls.
Kinjo *et al.* (13)	2004	38	31 superficial TCC, 7 invasive TCC	MUC7	RT-PCR			■	No expression in controls. 47.7% MUC7+ in superficial TCC, 77% MUC7 + in invasive disease. 42% of patients MUC7 +evolve to metastatic disease.

Table II continued

Table II continued

Authors	Year	No. of Patients	Stage of TCC studied	Marker	Technique	Samples			Results
						Blood	Nodes	Bone marrow	
Okegawa *et al.* (23)	2004	108	82 superficial TCC, invasive TCC. 26 invasive TCC	UPII, CK20	RT-PCR			■	No expression in controls. UPII+ and CK20+ associated with disease-free survival in multivariate analysis in superficial TCC but not in invasive TCC.
Ribal *et al.* (present series)	2004	66	57 invasive TCC, 9 superfical TCC	CK20	RT-PCR	■	■	■	33% and 16% of invasive bladder cancer CK20+ in blood and bone marrow samples respectively. 70% of invasive TCC CK20+ in lymph node. No CK20 expression in controls. No CK20 expression in non-invasive TCC. Correlation between CK20 in lymph node and pathological stage.

these, however, has simultaneously examined both hematogenous and lymphatic dissemination in bladder cancer. Moreover, to our knowledge, CK20 expression has not been used for detecting lymph node micrometastases in patients with TCC.

Specificity of CK20 as a marker for TCC. The specificity of CK20 as a marker for TCC remains controversial, since some reports have shown CK20 expression in the peripheral blood from patients with no history of bladder cancer (18-20). It is possible that these discrepancies could be due to the use of whole blood preparations for the detection of disseminated tumor cells, inappropriate for the intended purpose.

False-positive CK20 findings of RT-PCR assays can be avoided by analyzing peripheral blood mononuclear cells (PBMN) isolated by the Ficoll-Isopaque density-gradient centrifugation instead of analyzing whole preparations, which contain CK20-expressing granulocytes (2).

Using PBMN cells for the analysis of disseminated tumor cells, CK20 expression could not be detected in any of the 22 negative control blood samples or in the 5 bone marrow aspirates from patients suffering from a non-bladder pathology. CK20 transcripts were also undetectable in 5 lymph nodes of the 5 patients with prostate carcinoma.

A true comparison between the various studies performed regarding CK20 expression is difficult because of obvious differences in the methods employed. Selection of the PCR primers, the number of PCR cycles, the number of PCR reactions performed for each sample and the use of nested or single PCR assays are not standardized in the different studies. These divergences could explain the different sensitivities and specificities reported for the assay in the literature. In our study, two PCR for each sample were performed and one primer of the PCR assay was used in reverse transcription. Different primers were assayed and random primers were used in reverse transcription before making a decision about PCR conditions, and these assay conditions were rejected because CK20 expression was detected in the control samples. In our experience, the mRNA CK20 RT-PCR assay, performed under the conditions described, avoided the CK20 background expression detected in the control samples and reported by some authors.

In our experience, CK20 is a specific bladder marker since no CK20 expression was detected in the control samples. On the other hand, we could demonstrate CK20 expression in normal bladder and bladder tumor biopsies, according to the specific distribution of CK20 described in the literature. Despite the latest controversies about using CK20 as a marker of circulating cells, in our hands CK20 RT-PCR demonstrated good specificity for detecting circulating cells, since none of the control samples tested showed CK20 expression, while all the urothelial and tumors samples tested did.

Detection of minimal residual disease in lymph nodes by RT-PCR for CK20 mRNA. Lymph node metastases are one of the most important standard risk factors for patients with TCC when no evidence of systemic disease is present. However, routine histopathological examination of the lymph nodes

underestimates the prevalence of such metastases. In contrast, the detection of minimal residual disease in the lymph nodes of patients with node-negative cancer has been shown to be an important prognostic factor for many diseases, such as breast cancer, lung cancer and melanoma (6).

It has recently been demonstrated that the extent of lymphadenectomy was relevant to the survival of patients with invasive bladder cancer who had undergone cystectomy. It has also been postulated that the improved survival observed even in patients with lymph node-negative histopathology, who underwent more comprehensive lymphadenectomy, may be attributable to the removal of micrometastatic disease that was not identified (21).

In our study, we found that all but one of the histologically-positive lymph nodes were detected by the RT-PCR analysis. This indicates that RT-PCR for CK20 mRNA has a sensitivity at least equivalent to the conventional pathological analysis of nodes, since the positive results of both histological and molecular analysis matched in 95.8% of the patients studied. It is, however, important to point out that we observed a good correlation between lymph node CK20 RT-PCR and standard pathological risk factors, such as pathological stage ($p=0.04$). Although the presence of CK20 mRNA in lymph nodes can also be the expression of circulating bladder cancer cells in blood capillaries as they migrate through these tissues, it seems more reasonable that bladder cancer cells reach the lymph nodes *via* active migration through lymphatic channels. This capability *per se* indicates a malignant characteristic and may partly explain the good correlation of lymph node RT-PCR with pathological stage.

Similar results for sensitivity were achieved using UPII as a marker in the only study on lymph node molecular staging previously reported (14). This paper also demonstrated 50% positivity in nodal tissue by the uroplakin II (UPII) RT-PCR assay, while only 15% of patients showed pathological node involvement. Similar figures were found in our series, where 46.6% of the histopathologically-negative lymph nodes expressed CK20 according to the RT-PCR assay. This rate of detection of CK20 expression in lymph nodes is in agreement with previously reported loco-regional recurrence in patients with invasive bladder cancer who underwent radical cystectomy(1-22), and with rates of micrometastases detected in the lymph nodes at thorough re-examination of the excised lymph nodes (21). Longer follow-up of these patients is necessary to establish the real meaning of CK20 expression in lymph nodes.

Detection of circulating cells in invasive TCC. The presence of circulating cancer cells, from patients with solid tumors detected by molecular analysis, remains controversial since no specific marker for circulating cells in each tumor has been established and the real import of these circulating cells is still unclear. The main findings published on the detection of tumor cells in peripheral blood and bone marrow from bladder cancer patients are summarized in Table II.

In order to avoid false-positive results in blood sample analysis, two blood samples were collected from each patient and RT-PCR analysis was carried out only on the second one, with the purpose of avoiding Merkell cell contamination of the sample.

CK20-positive cells were demonstrated in 19-35% patients with localized muscle-invasive bladder cancer (2, 4, 5, 10, 23) and in 63-83% patients with metastatic disease (4). According to these results, CK20 expression was identified in the peripheral blood from 19 out of the 57 (33%) patients who underwent radical cystectomy, as well as in the bone marrow samples from 4 out of 21 patients (16%). It is important to point out that CK20 transcripts were detected in 3 out of 4 (75%) blood samples from patients with metastatic disease.

CK20 transcripts were also detected in 7 out of 17 (41%) patients with T2 TCC, as well as in 12 out of 29 (41%) patients with T3-T4 TCC, in a definitive pathological report. These ranges of circulating cancer cells are in accordance with the rate of metastatic disease in patients with muscle-invasive disease, but with no evidence of clinical metastasis even after radical cystectomy. Obviously, further evaluation of the real meaning of CK20 expression in peripheral blood from patients with bladder cancer is warranted.

None of the patients with superficial bladder cancer included in our series showed CK20 expression in peripheral blood, except for one patient with T1G3 TCC with associated Cis and BCG therapy failure and formal contraindication for radical surgery. These findings are in agreement with other findings reported in the literature (4, 5, 7, 9). Some authors reported detection of circulating cancer cells in superficial TCC, ranging from 10%-47% (8, 13). Interestingly, a recent report of a multivariate analysis for postoperative recurrence in patients with superficial bladder cancer showed that nested RT-PCR for UPII and CK20 was significantly associated with disease-free survival (23). To our knowledge, this is the first time that two dissemination routes (hematogenous and lymphatic) have been studied in the same cohort of patients. In this connection, we observed that 96.3% of patients who showed CK20 expression in the peripheral blood showed, at the same time, CK20 expression in lymph node samples, while 44% of the patients showed CK20 expression in lymph nodes and undetectable expression in the blood. These findings suggest that lymphatic dissemination occurs early in metastatic progression, prior to hematogenous dissemination.

Conclusion

The CK20 RT-PCR assay is a reliable assay to detect pathologically-positive lymph nodes. Additionally, this approach detects CK20 expression in microscopically-negative lymph node samples. Lymph node CK20 RT-PCR seems to

correlate better with pathological stage than blood RT-PCR. It is important to point out the specificity of the CK20 expression and the lack of detection in the control population, which strongly suggest that the lymph node RT-PCR assay actually detects bladder cancer tumor cells. Using the CK20 RT-PCR assay, the presence of circulating bladder cancer cells could be demonstrated in 33% of patients with muscle-invasive TCC. It is necessary to further evaluate the significance of CK20 as a molecular marker for staging in patients with bladder cancer and only long-term follow-up of these patients will determine the clinical significance of these findings.

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