Detection of Micrometastases in Pelvic Lymph Nodes in Patients Undergoing Radical Cystectomy for Focally Invasive Bladder Cancer by Real-time Reverse Transcriptase-PCR for Cytokeratin 19 and Uroplakin II

Toshifumi Kurahashi,[1,2] Isao Hara,[1] Nobutoshi Oka,[2] Sadao Kamidono,[1] Hiroshi Eto,[3] and Hideaki Miyake[3]

Abstract

Purpose: The objective of this study was to clarify the significance of micrometastases in pelvic lymph nodes in patients who underwent radical cystectomy for bladder cancer.

Experimental Design: We included 40 patients with locally invasive bladder cancer who underwent radical cystectomy and pelvic lymphadenectomy. Expression of cytokeratin 19 (CK19), uroplakin II (UP II), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in 760 lymph nodes were assessed by a fully quantitative real-time reverse transcription-PCR (RT-PCR) assay. The quantification value of CK19 or UP II mRNA was described as each value relative to GAPDH mRNA. In this study, we regarded specimen in which either CK19 or UP II mRNA was positive as "presence of micrometastasis."

Results: Routine pathologic examinations detected tumor cells in 29 lymph nodes from six patients. Real-time RT-PCR identified positive expression of CK19 and UP II mRNAs in 49 lymph nodes from 10 patients and 98 lymph nodes from 16 patients, respectively. Of 633 lymph nodes from 34 patients with no pathologic evidence of nodal involvement, 13 nodes from five patients and 58 nodes from 10 patients were diagnosed as positive for CK19 and UP II mRNAs expression, respectively, by real-time RT-PCR. Presence of micrometastases was significantly associated with other conventional prognostic variables, including pathologic stage and microvascular invasion. Disease recurrence was occurred in eight patients, among whom four patients were negative for lymph node metastasis by routine pathologic examination and diagnosed as having micrometastasis by real-time RT-PCR assay. Furthermore, cause-specific survival rate in patients without micrometastasis was significantly higher than that in those with micrometastasis, irrespective of the presence of pathologic-positive nodes.

Conclusions: Approximately 30% of locally invasive bladder cancer shed cancer cells to pelvic lymph nodes, and disease recurrence after radical cystectomy could be explained, at least in part, by micrometastases in pelvic lymph nodes.

Radical cystectomy coupled with a pelvic lymphadenectomy has been regarded as the most effective treatment for locally invasive bladder cancer. Indeed, contemporary series report that this surgery cures most muscle-invasive tumors confined to the bladder wall and approximately half that have spread into the perivesical fat (1–3), but despite the presence of subpopulation showing long-term survival, lymph node metastasis is definitively an unfavorable sign suggesting poor prognosis (1–5). However, routine microscopic examination of lymphadenectomy specimens can miss small cancer foci, and these findings may be partially accounted for the presence of histologically undetectable micrometastases in the regional lymph nodes. In fact, higher sensitivity for detecting micrometastatic cancer cells in surgically removed regional lymph nodes can be achieved by several molecular and histologic techniques in patients with a various types of malignant tumors, such as prostate, breast, and cervical cancers (6–9), but to date, the application of such techniques for detecting bladder cancer cells in pathologically uninvolved lymph nodes has not been well investigated, resulting in the lack of available information of micrometastasis of bladder cancer. Collectively, these findings suggest that a novel approach for analyzing micrometastatic features of bladder cancer cells in the pelvic lymph nodes needs to be identified.

Recently, a real-time detection and quantitative PCR-based assay has been developed (10). The advantage of this technique is the specific detection of rare events; that is, the sensitivity has been shown to allow for the detection of 10 to 100 pg of RNA from target gene. Furthermore, it is highly reproducible and quantitative, significantly eliminates the risks of contamination

Authors' Affiliations: [1]Department of Urology, Kobe University School of Medicine and [2]Department of Urology, Hara Genitourinary Hospital, Kobe, Japan, and [3]Department of Urology, Hyogo Medical Center for Adults, Akashi, Japan

Received 11/10/04; revised 2/26/05; accepted 3/1/05.

Grant support: Ministry of Education, Science and Culture of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Hideaki Miyake, Department of Urology, Hyogo Medical Center for Adults, 13-70 Kitaohji-cho, Akashi 673-8558, Japan. Phone: 81-78-929-1151; Fax: 81-78-929-2380; E-mail: hideakimiyake@hotmail.com.

encountered with other conventional PCR-based assays, and requires no post-PCR product manipulations. Therefore, this method has become widely used for detecting occult micrometastatic tumor cells in the resected lymph node specimens (9, 11–14). For example, Van Trappen et al. reported, using real-time reverse transcription-PCR (RT-PCR) targeting cytokeratin 19 (*CK19*) gene, that about 50% of early-stage cervical cancer shed tumor cells to the pelvic nodes, and the amount of *CK19* expression was related to clinicopathologic features (9). Considering these findings, it would be attractive to use real-time RT-PCR assay as the tool for clarifying the significance of micrometastases in the pelvic lymph node from patients undergoing radical cystectomy.

Few attempts have been made to detect genes whose expression is exclusively restricted to normal utothelium and/or urothelial cancer. Recently, genes that encode urothelium-specific transmembrane proteins, uroplakins, were cloned, and the expression of uroplakins is highly specific to urothelium and preserved in neoplasm of urothelial origin (15). Furthermore, the usefulness of a nested RT-PCR targeting uroplakin II (UP II) for detecting circulating urothelial cancer cells has previously been reported (16, 17). In the present study, we did a fully quantitative real-time RT-PCR assay targeted against CK19, which is the conventional marker for epithelial cells (9), in addition to UP II in 760 fresh pelvic lymph nodes obtained from 40 patients who underwent radical cystectomy for locally invasive bladder cancer and analyzed the clinical significance of occult micrometastasis of bladder cancer cells in pelvic lymph nodes.

Patients and Methods

Surgical specimens. This study was approved by the research ethics committee of our institution, and the informed consent was obtained from all patients at the time of enrollment. Lymph node specimens were obtained from 40 patients with locally invasive bladder cancer who underwent radical cystectomy and pelvic lymphadenectomy between October 2001 and July 2004. Pelvic lymphadenectomy was done targeting the obturator fossa and external iliac region by removing all fatty, connective, and lymphatic tissues. Lymph node samples were available from three patients without any evidence of having malignant diseases. Each lymph node was bisected. One half was snap-frozen immediately and stored at −80°C until assessed, and the remainder was fixed in formalin, embedded in paraffin, and stained with H&E for histopathologic examinations. The tumor stage and grade were examined according to the tumor-node-metastasis classification (18) and the WHO system (19), respectively, by a single pathologist.

Real-time reverse transcription-PCR assay. Total RNA was extracted from the lymph node specimens using the acid guanidinium isothiocyanate, phenol chloroform method (20), and 1 μg of each total RNA was reverse transcribed using an Oligo dT and Superscript preamplification system (Life Technologies, Rockville, MD). To analyze the expression levels of CK19, UP II, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs, real-time quantitative PCR was done using Sequence Detector (ABI PRISM 7700, PE Applied Biosystems, Foster City, CA). Sequences of primers and probes for these genes were determined by Primer Express software (PE Applied Biosystems). Selected sequences of forward and reverse primers, and probes are shown in Table 1. The probes used in this study consisted of an oligodeoxynucleotide with a 6-carboxy-fluorescein reporter dye and 6-carboxy-tetramethylrhodamine quencher dye. Each cDNA was analyzed by quantitative PCR in a 50 μL volume using Master Mix (PE Applied Biosystems), including PCR buffer, $MgCl_2$, dATP, dCTP, dGTP, dUTP, AmpErase UNG, and AmpliTaq Gold DNA polymerase. The condition of thermal cycling was 50 cycles of amplification consisting of 15 seconds at 95°C and 1 minute at 60°C.

Table 1. Primer and probe sequences for real-time RT-PCR

Target gene	Primers (5′-3′)	Probe (5′-FAM/TAMRA-3′)
CK19	Forward, TCGACAACG CCCGTCTG	CCGAACCAAGTTT GAGACGG AACAGG
	Reverse, CCACGCTCAT GCGCAG	
UP II	Forward, TGGAGCCCC TCTTCT GTAAGTC	ATACTCACTGA GGGAACTCT ACTCTCTCCC AAACC
	Reverse, CAGTGGCTGTCCCC TTCTTC	
GAPDH	Forward, GAAGGTGAAGGT CGGAGTC	CAAGCTTCCCGTT CTCAGCC
	Reverse, GAAGATGGTGAT GGGATTTC	

Real-time quantitation was done based on Taqman assay according to the manufacture's instruction as described previously (21). After the generation of a real-time amplification plot based on normalized fluorescence signal, the threshold cycle (C_t), which is the fractional cycle number at which the amount of amplified target reached a fixed threshold, was determined. The C_t was then used for kinetic analysis and was proportional to the initial number of target copies in the sample. The starting quantity of a sample was calculated after comparison with the C_ts of a serial dilution of a positive control, human bladder cancer KoTCC-1 cells, which were established in our laboratory (22).

Both the precise amount and quality of total RNA added to each reaction mix are extremely difficult to assess; therefore, transcripts of the *GAPDH* gene were quantified as an internal reference according to a quantitative PCR assay, and each sample was normalized based on its GAPDH content. The quantification value of CK or UP II mRNA was described as each value relative to GAPDH mRNA. To exclude false positives, we used the mean relative mRNA value plus 2 SDs of CK19 or UP II mRNA expression in 53 lymph nodes from three patients without any evidence of having malignant diseases as the cutoff value of CK19 or UP II, respectively, and values above the cutoff value of CK19 or UP II mRNA were defined as CK19 or UP II mRNA positive, respectively. In this study, we regarded specimen in which either CK19 or UP II mRNA was positive as "presence of micrometastasis."

Statistical analyses. Differences between the two groups were compared using the χ^2 test or Mann-Whitney *U* test. The cause-specific survival rates were calculated by the Kaplan-Meier method, and the difference was determined by log-rank test. Factors associated with cause-specific survival were analyzed by Cox's proportional hazards regression model. All statistical calculations were done by use of the Statview 5.0 software (Abacus Concepts, Inc., Berkeley, CA), and *P*s < 0.05 were considered significant.

Results

Expression of GAPDH mRNA in all lymph node specimens was confirmed. In 53 lymph nodes from three patients without any evidence of malignant diseases, the mean values of relative CK19 and UP II mRNAs expression plus 2 SDs were 0.3 and 1.8, respectively, and these values were used as cutoff points for positive expression of CK19 and UP II mRNA in lymph nodes from patients with bladder cancer in the subsequent study. Real-time RT-PCR assays in 760 pelvic lymph nodes from patients with locally invasive bladder cancer detected various amounts of relative expression levels of CK19 and UP II mRNAs (CK19: mean 0.8, range 0-9.6; UP II: mean 2.1, range 0-9.8).

Routine pathologic examinations detected tumor cells in 29 lymph nodes from six patients, and among these 29 nodes, real-time RT-PCR confirmed the positive expression of CK19 and UP II mRNAs in 27 nodes from five patients and all 29 nodes from six patients, respectively. In these six patients, positive CK19 and UP II mRNAs expression were detected in additional 7 and 11 histologically uninvolved lymph nodes, respectively; thus, a total of 34 and 40 nodes were diagnosed as positive for CK19 and UP II mRNAs expression by real-time RT-PCR assay. Of the 633 nodes from the remaining 34 patients without histologic evidence of pelvic lymph node metastases, positive CK19 and UP II mRNAs expression were detected in 13 nodes from five patients and 58 nodes from 11 patients, respectively. Among these nodes, 60 from 12 patients were judged to be positive for either CK19 or UP II mRNA expression; therefore, these 12 patients were regarded as having micrometastases to pelvic lymph nodes. These outcomes are summarized in Table 2 by dividing 40 patients into the following three groups; that is, six with histologically detected lymph node metastases (group A), 12 with micrometastases despite the lack of histologic evidence of nodal involvement (group B), and the remaining 22 without any findings of lymph node metastases by both histologic and real-time RT-PCR analyses (group C).

The incidence of micrometastases according to anatomic location was analyzed. As shown in Table 3, similar metastatic patterns of bladder cancer cells to external iliac region and/or obturator fossa was observed between groups A and B irrespective of the presence of histologically confirmed nodal involvement. We further compared clinicopathologic features among these three groups. As shown in Table 4, despite the no significant differences in several factors examined between groups A and B, pathologic stage and incidence of microvascular invasion in groups A and B were significantly greater than those in group C ($P < 0.05$ and $P < 0.01$, respectively).

Table 2. Outcomes of histologic examination and real-time RT-PCR assay

Group	A	B	C
No. patients	6	12	22
No. dissected lymph nodes	127	226	407
Histologic examination			
No. positive patients	6	0	0
No. positive lymph nodes	29	0	0
Real-time RT-PCR assay for CK19			
No. positive patients	5	5	0
No. positive lymph nodes	34	13	0
Real-time RT-PCR assay for UP II			
No. positive patients	6	11	0
No. positive lymph nodes	40	58	0
Micrometastasis			
No. positive patients	6	12	0
No. positive lymph nodes	44	60	0

Table 3. Lymph node metastases detected by histologic examination and real-time RT-PCR assay according to anatomic location

Group	A	B	C
No. patients	6	12	22
No. positive lymph nodes detected by histologic examination			
External iliac region	12	0	0
Obturator fossa	17	0	0
No. positive lymph nodes detected by real-time RT-PCR assay			
External iliac region	19	25	0
Obturator fossa	25	35	0

The median follow-up period of the 40 patients included in this study was 22 months (range, 7-36 months). In this series, disease recurrence occurred in three patients, four patients, and one patient in group A, B, and C, respectively (Table 5). The median interval between radical cystectomy and disease recurrence in groups A, B, and C were 6, 7, and 13 months, respectively. As shown in Fig. 1, cause-specific survival rates in groups A and B were significantly lower than that in group C ($P < 0.005$: group A versus group C and $P < 0.05$: group B versus group C). However, multivariate analysis using Cox's proportional hazards regression model showed that the presence of micrometastasis detected by real-time RT-PCR assay could not be an independent predictor for cause-specific survival (data not shown). Of the 22 patients with pathologically organ-confined disease (i.e., pT2 ≤ and pN0), only two underwent disease recurrence who were diagnosed as having micrometastases in pelvic lymph nodes by real-time RT-PCR assays.

Discussion

Despite the most effective treatment for patients with invasive bladder cancer, radical cystectomy with pelvic lymph node dissection generally provides a 5-year survival of <70% (1–3). The etiology of disease recurrence after successfully done radical surgery is likely multifactorial; however, considering the finding that ~30% of patients without pathologic evidence of positive surgical margin as well as nodal involvement will suffer disease recurrence, a significant proportion of these recurrence may be due to occult metastases to pelvic lymph nodes undetected by routine pathologic examinations. To our knowledge, there seem few studies assessing whether microscopic foci of bladder cancer cells is present in histologically uninvolved pelvic nodes using molecular approaches; therefore, the clinical significance of micrometastases in pelvic nodes remains obscure. Because accurate staging of invasive bladder cancer provides the ability to predict therapeutic outcomes and tailor appropriate adjuvant therapies to the individual patient, we

Table 4. Comparison of conventional prognostic indicators according to lymph node metastases detected by histologic examination and real-time RT-PCR assay

Group	A	B	C
No. patients	6	12	22
Gender			
Male	5	10	19
Female	1	2	3
Pathologic stage			
pT2 or less	1	4	4
pT3 or more	5	8	18
Grade			
G2	0	1	2
G3	6	11	20
Microvascular invasion*			
Negative	1	3	6
Positive	5	9	16
Concomitant carcinoma *in situ*			
Negative	2	2	4
Positive	4	10	18

*Presence of either blood vessel invasion or lymphatic invasion.

Table 5. Incidence of disease recurrence according to lymph node metastases detected by histologic examination and real-time RT-PCR assay

Group	A	B	C
No. patients	6	12	22
No. patients undergoing disease recurrence (%)	3 (50.0)	4 (33.3)	1 (4.5)
Median time to disease recurrence after radical cystectomy (mo)	6	7	13
Pathologically organ-confined disease*			
No. patients undergoing disease recurrence/total no. patients	0/0	2/4	0/18
Pathologically extravesical disease†			
No. patients undergoing disease recurrence/total no. patients	3/6	2/8	1/4

*pT2 ≥ and pN0.
† pT3 ≥ or pN1.

investigated CK19 and UP II mRNAs expression in 760 pelvic lymph nodes dissected at radical cystectomy from 40 patients with clinically localized prostate cancer by a quantitative real-time RT-PCR assay and analyzed various clinicopathologic factors according to the findings on this assay.

In this series, we did standard pelvic lymphadenectomy targeting the external iliac region and obturator fossa for all 40 patients, and the mean number of lymph nodes removed at surgery in these patients was 19.0. Based on an autopsy study, ~20 lymph nodes have been shown to serve as a guideline for optimal and representative pelvic lymph node dissection (23), suggesting that the procedure for pelvic lymphadenectomy done in this study, which met this requirement, would be suitable. Fifty-three lymph nodes from three patients without the evidence of malignant disease were also included and used for determining the cutoff points for positive expression of CK19 and UP II mRNAs by real-time RT-PCR. To avoid overestimating the significance of micrometastases of bladder cancer cells, the mean relative mRNA value plus 2 SDs of CK19 or UP II mRNA expression in these 53 nodes were used as the cut off value for positive expression of CK19 or UP II mRNA, respectively. Furthermore, we evaluated expression levels of both CK19 and UP II mRNAs (9, 16, 17) in all nodes to prevent from overlooking occult micrometastatic foci in lymph nodes. Collectively, these findings suggest that the present study was carried out under the rigorous and ideal conditions, which contributes to enhance the reliability of the current outcomes.

In this series, among 633 lymph nodes from 34 patients without pathologic evidence of nodal involvement, 60 nodes from 12 patients were diagnosed as the presence of occult micrometastasis by real-time RT-PCR assay. Theses findings suggest that ~30% of locally invasive bladder cancer may shed cancer cells to pelvic lymph nodes. Furthermore, characterization of clinicopathologic features according to nodal status showed that there are no significant differences in several conventional prognostic factors between patients with histologically detected nodal involvement and those with nodes positive for micrometastases despite the lack of histologically positive findings. Anatomic locations of micrometastatic nodes were also similar between these two patients groups. These findings strongly suggest that even if the lack of histologic confirmation, some of micrometastatic disease diagnosed by the current real-time RT-PCR assay may have a similar biological character to histologically positive nodal disease. This hypothesis was supported by the incidence of disease recurrence following radical cystectomy; that is, although the follow-up period of this study is too short to draw the conclusion concerning the matter associated with disease recurrence, there were no significant differences in the

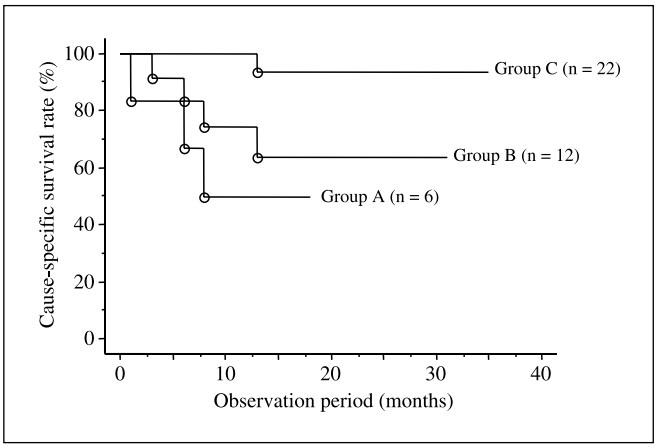

Fig. 1. Comparison of cause-specific survival rates in groups A, B, and C by the Kaplan-Meier method. The cause-specific survival rates in groups A and B were significantly lower than that in group C ($P < 0.005$, group A versus group C; $P < 0.05$, group B versus group C by the log-rank test).

incidence of disease recurrence between these two groups. In addition, it also supports this hypothesis that disease recurrence occurred in two patients with pathologically organ confined disease who diagnosed as positive for micrometastases.

To further address the significance of micrometastases in invasive bladder cancer, there are several problems to be elucidated. For example, it should be analyzed whether the CK19 and UP II levels in the primary tumors affect the present outcomes. More appropriate cutoff points should be determined based on the findings from larger number of control samples. It is of interest whether histologically undetectable or dormant micrometastatic disease in the lymphatic system will always progress in clinically significant recurrence after variable disease-free recurrence. If not, it will be necessary to establish the diagnostic system differentiating significant micrometastatic diseases from insignificant diseases. In addition, recent studies have reported the prognostic benefit of lymphadenectomy in subpopulation of patients with pathologically positive nodes who underwent radical cystectomy (4, 5). If there is a significant effect of pelvic lymph node dissection on survival for such patients category, it would be interesting to evaluate the effect of removing micrometastatic nodes on the prognosis. Assessment of these issues may make it possible to determine the more appropriate procedure of lymphadenectomy considering the findings of molecular staging. Finally, we should consider how to introduce molecular staging into routine clinical practices; that is, it will be required to develop more simple technique maintaining sensitivity similar to the current real-time RT-PCR assay.

The results of this study showed the usefulness of the quantitative real-time RT-PCR targeting the expression of *CK19* and *UP II* genes for identifying mictometastatic tumor foci in pelvic lymph nodes from locally invasive bladder cancer at radical cystectomy. Although longer follow-up periods will be absolutely necessary to draw the definitive conclusion, the present findings suggest that some of micrometastases diseases in pelvic lymph nodes may, at least in part, cause disease recurrence after radical cystectomy.

References

1. Stein JP, Lieskovsky G, Cote R, et al. Radical cystectomy in the treatment of invasive bladder cancer: long-term results in 1,054 patients. Int J Clin Oncol 2001;19:666–75.
2. Nishiyama H, Habuchi T, Watanabe J, et al. Clinical outcome of a large-scale multi-institutional retrospective study for locally advanced bladder cancer: a survey including 1131 patients treated during 1990-2000 in Japan. Eur Urol 2004;45:176–81.
3. Wade M, Seigne JD. Surgical management of bladder cancer in 2003. Expert Rev Anticancer Ther 2003;3:781–92.
4. Vieweg J, Gschwend JE, Herr HW, et al. Pelvic lymph node dissection can be curative in patients with node positive bladder cancer. J Urol 1999;161:449–54.
5. Poulsen AL, Horn T, Steven K. Radical cystectomy: extending the limits of pelvic lymph node dissection improves survival for patients with bladder cancer confined to the bladder wall. J Urol 1998;160:2015–20.
6. Okegawa T, Nutahara K, Higashihara E. Detection of micrometastatic prostate cancer cells in the lymph nodes by reverse transcriptase polymerase chain reaction is predictive of biochemical recurrence in pathological stage T2 prostate cancer. J Urol 2000;163:1183–8.
7. Potter SR, Mangold LA, Shue MJ, et al. Molecular and immunohistochemical staging of men with seminal vesicle invasion and negative pelvic lymph nodes at radical prostatectomy. Cancer 2000;89:2577–86.
8. Noguchi S, Aihara T, Nakamori S, et al. The detection of breast carcinoma micrometastases in axillary lymph nodes by means of reverse transciptase-polymerase chain reaction. Cancer 1994;74:1595–600.
9. Van Trappen PO, Gyselman VG, Lowe DG, et al. Molecular quantification and mapping of lymph-node micrometastases in cervical cancer. Lancet 2001;357:15–20.
10. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996;6:995–1001.
11. Mrras M, Mikhitarian K, Walters C, et al. Quantitative real-time RT-PCR detection of breast cancer micrometastasis using a multigene marker panel. Int J Cancer 2001;93:162–71.
12. Yoshioka S, Fujiwara Y, Sugita Y, et al. Real-time reverse transcriptase-polymerase chain reaction for intraoperative diagnosis of lymph node micrometastasis: clinical application for cervical lymph node dissection in esophageal cancers. Surgery 2002;132:34–40.
13. Kubota K, Nakanishi H, Hiki N, et al. Quantitative detection of micrometastases in the lymph nodes of gastric cancer patients with real-time RT-PCR: a comparative study with immunohistochemistry. Int J Cancer 2003;105:136–43.
14. Inokuchi M, Ninomiya I, Tsugawa K, Terada I, Miwa K. Quantitative evaluation of metastases in axillary lymph nodes of breast cancer. Br J Cancer 2003;89:1750–6.
15. Wu XR, Lin JH, Walz T, et al. Mammalian uroplakins. A group of highly conserved urothelial differentiation-related membrane proteins. J Biol Chem 1994;269:13716–24.
16. Lu JJ, Kakehi Y, Takahashi T, et al. Detection of circulating cancer cells by reverse transcription-polymerase chain reaction for uroplakin II in peripheral blood of patients with urothelial cancer. Clin Cancer Res 2000;6:3166–71.
17. Li SM, Zhang ZT, Chan S, et al. Detection of circulating uroplakin-positive cells in patients with transitional cell carcinoma of the bladder. J Urol 1999;162:931–5.
18. Sobin LH, Wittekind CH, editors. TNM classification of malignant tumors. 5th ed. New York: Wiley-Liss; 1997.
19. Mostofi FK, Davis CJ, Sesterhenn IA. Histological typing of urinary bladder tumors. 2nd ed. Berlin: Springer Verlag; 1999.
20. Chomczynski P, Saachi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
21. Das H, Koizumi T, Sugimoto T, et al. Quantitation of Fas and Fas ligand gene expression in human ovarian, cervical and endometrial carcinomas using real-time quantitative RT-PCR. Br J Cancer 2000;82:1682–8.
22. Miyake H, Hara I, Kamidono S, Gleave ME. Synergistic chemosensitization and inhibition of tumor growth and metastasis by the antisense oligodeoxynucleotide targeting clusterin gene in a human bladder cancer model. Clin Cancer Res 2001;7:4245–52.
23. Weingartner K, Ramaswamy A, Bittinger A, Gerharz EW, Voge D, Riedmiller H. Anatomical basis for pelvic lymphadenectomy in prostate cancer: results of an autopsy study and implications for the clinic. J Urol 1996;156:1969–73.

Clinical Cancer Research

Detection of Micrometastases in Pelvic Lymph Nodes in Patients Undergoing Radical Cystectomy for Focally Invasive Bladder Cancer by Real-time Reverse Transcriptase-PCR for Cytokeratin 19 and Uroplakin II

Toshifumi Kurahashi, Isao Hara, Nobutoshi Oka, et al.

Clin Cancer Res 2005;11:3773-3777.