Advances in Brief

p14ARF Promoter Hypermethylation in Plasma DNA as an Indicator of Disease Recurrence in Bladder Cancer Patients[1]

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Abstract

Purpose: **Several genes are reported to be implicated in bladder carcinogenesis, including *p53*, *p16INK4a*, *pRb*, *erbB-2*, *Cyclin D1*, *H-ras*, *EGFR,* and *c-myc*. Gene alterations in plasma DNA identical to those observed within the tumor have been detected in various types of neoplasia.**

Experimental Design: **We analyzed loss of heterozygosity in six microsatellite markers (D17S695, D17S654, D13S310, TH2, D9S747, and D9S161), *p53* and K-*ras* mutations, and the promoter status of *p14ARF* and *p16INK4a* in the mononuclear normal blood cells, tumor, and plasma DNA of 27 bladder cancer patients. We also studied the distribution of several clinicopathological parameters in these patients in regard to molecular alterations.**

Results: **Seventeen (63%) cases displayed the same alteration in plasma and tumor DNA (some patients showed more than one alteration simultaneously). Plasma *p14ARF* promoter hypermethylation was associated with the presence of multicentric foci ($P = 0.03$), larger tumors ($P = 0.01$), and relapse of the disease ($P = 0.03$). Plasma loss of heterozygosity was also linked to disease recurrence ($P = 0.02$).**

Conclusions: **The results indicate that *p14ARF* aberrant promoter methylation could be involved in bladder carcinogenesis and that plasma DNA is a potential prognostic marker in urinary bladder cancer.**

Introduction

Urinary bladder cancer is the fifth most common tumor in Western societies. Its occurrence increases with age and it is greater in men (with a sex ratio between 3:1 and 4:1; Ref. 1). The risk of bladder cancer is doubled in smokers (2), but there are other risk factors (3). About 95% of bladder neoplasms are transitional cell carcinomas. The remainder are squamous tumors, adenocarcinomas, and other subtypes. At the time of diagnosis, >60% of the transitional carcinomas are papillary noninvasive (Ta), 10–20% show invasion limited to the lamina propria (T1), and 20% present muscle or deeper infiltration (T2-T4; Refs. 4, 5). Between 15 and 30% of bladder tumors show grade and stage progression (3, 6). Despite radical local therapy, half the patients with muscle-invasive bladder tumors die from metastatic disease (7). Bladder cancer is the result of monoclonal genetic changes, and the multiple synchronous or metachronous tumors are derived from micrometastatic foci that have migrated from the original site rather than from a polyclonal mutation (8–10).

Many studies have sought predictive markers in bladder cancer patients. Several genes are involved, including *erbB-2*, *EGFR*, *c-myc*, *Cyclin D1*, *H-ras*, *p53*, *p16INK4a,* and *pRb*. Frequent deletions at 2q, 3p, 4q, 5q, 6q, 8p, 9p, 9q, 11p, 11q, and 13q, and frequent gains of 1q, 3q, 5p, 6p, chromosome 7, 8q, 17q, and 20q may pinpoint the location of other, yet unidentified tumor suppressor genes and oncogenes with a role in bladder cancer (11–15).

Free-circulating tumor DNA has been detected in peripheral blood of both healthy subjects and tumor patients. The mean concentration of soluble DNA in plasma was 14 ng/ml in control subjects (16) and 180 ng/ml in cancer patients (17). Gene alterations identical to those observed within the tumor have been detected by PCR in the plasma DNA of patients with various types of tumor (18–29). The prognostic implications of circulating plasma DNA in patients with malignancies regarding the outcome of the disease are being examined. A correlation with poor survival in pancreatic carcinomas has been reported (30), and two studies about the follow-up of lung cancer and melanoma patients have been published recently (31, 32). A novel plasma DNA utility has been used to screen somatic mutations that are frequent in tumors and detect them in a preclinical phase of the disease (33).

The presence of microsatellite alterations in plasma DNA of bladder cancer patients has been reported recently (34, 35). The present study aims to: (*a*) detect tumor DNA at diagnosis in the plasma of such patients; and (*b*) analyze the distribution of various clinicopathological parameters. We characterized tumor DNA in plasma by checking alterations in microsatellite markers at described deleted loci, in tumor suppressor genes, and in the K-*ras* oncogene. To our knowledge, this is the first analysis of p14ARF promoter status in bladder cancer.

Materials and Methods

Tissue Sampling and DNA Extraction. Between April 1999 and June 2001, 27 samples from tumor tissue were ob-

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tained immediately after TUR[3] from patients with a diagnosis of bladder cancer (26 transitional cell carcinomas and 1 squamous tumor). They were then snap-frozen in liquid nitrogen and stored until processing. All of the specimens underwent histological examination (*a*) to confirm the diagnosis; (*b*) to confirm the presence of tumor and evaluate the percentage of tumor cells in each sample; and (*c*) to perform pathological staging. All of the samples showed at least 75% tumor cells. A blood sample was collected from each patient on the day of surgery before the TUR to avoid the possible clearance of plasma DNA after removal of the primary tumor. DNA was extracted from tumor tissues, normal blood cells, and plasma immediately. DNA was extracted from tumor tissue samples and peripheral blood mononuclear cells by a nonorganic method (5–4520 kit; Oncor, Gaithersburg, MD). Plasma DNA was purified on Qiagen columns (Quiamp Blood kit; Qiagen, Hilden, Germany) following the protocol for blood and body fluids, modified as described elsewhere (26).

Analysis of Clinicopathological Parameters. The following information was obtained from the medical records of the 27 patients: (*a*) birth and diagnosis dates; (*b*) radiological lymph node enlargement; (*c*) presence of multicentric disease; (*d*) tumor size; (*e*) histological subtype; (*f*) histological grade; (*g*) invasiveness; (*h*) pathological stage; (*i*) treatment; and (*j*) recurrences. All of the tumors were graded according to the number of mitotic elements and atypias. Pathological stage was classified by the Tumor-Node-Metastasis system, where T is the invasiveness of the tumor, N the lymph nodes affected, and M the metastases to other organs. All of the patients underwent TUR, and after staging, 9 patients were followed without any adjuvant treatment, 15 received intravesical therapy based on bacillus Calmette-Guérin, and 3 underwent radical cystectomy for invasion or progression of bladder cancer.

Microsatellite Analysis and PCR Conditions. PCR was performed in a 10-μl reaction volume containing 50 ng/μl of sample DNA (tumor, mononuclear blood cells, or plasma DNA), 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 1 μl of 10 × PCR buffer [100 mM Tris-HCl, (pH 8.3), 500 mM KCl] 200 μM dNTP, 0.6 μM of each primer, and a range of concentrations of KCl and $MgCl_2$ depending on the polymorphic marker. A 30-cycle amplification was carried out in a thermal cycler (Perkin-Elmer, Cetus, Foster City, CA). Six microsatellite markers were used to determine LOH on the following chromosomes: (*a*) 17: D17S695 (36) and D17S654 (37); (*b*) 13: D13S310 (38); (*c*) 11: TH2 (39); and (*d*) 9: D9S747 (accession no. GDB 335542) and D9S161 (40). Annealing was at 63°C, 65°C, 56°C, 58°C, 63°C, and 60°C, respectively. The alleles were separated by mixing the 10 μl of the PCR products with 10 μl of loading buffer (0.02% xylene cyanol and 0.02% bromphenol blue). Electrophoresis was run on nondenaturing 8% polyacrylamide gels for 3–5 h at 500 V at room temperature. The allelic band intensity was then detected by silver staining and analyzed by densitometry. The gel image was captured using a GS-690 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA), digitalized in 400 dpi, and analyzed using Multi-Analyst/PC (Bio-Rad Laboratories). LOH was accepted when the signal of the allele was >75% lower than that observed in the normal counterpart DNA, considering that variations in the amplification process and especially the presence of small amounts of normal cells in the tumor tissue or, in our study, of normal blood cell DNA in plasma DNA, could lead to the expression of various band intensities in the total absence of one allele.

Mutational Study of the *p53* Gene. To detect point mutations in the conserved exons of *p53*, PCR-SSCP was performed as described elsewhere (26). PCR was carried out in a final volume of 10 μl containing 50 ng of DNA template, 1 μl of 10 × PCR buffer, 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc.), 200 μM dNTP, 0.6 μM of each primer, and a range of concentrations of KCl and $MgCl_2$ depending on the exon amplified. For PCR amplifications, the samples underwent 30 cycles. The amplified products were denatured by adding 10 μl of denaturing stop solution, which contained 98% formamide, 10 ml/liter edathamil (pH 8.0), 0.02% xylene cyanol, and 0.02% bromphenol blue, heated to 95°C for 5 min and rapidly cooled on ice. Electrophoresis was run on nondenaturing 10% polyacrylamide gels for 12–15 h at 250 v. The allelic band intensity on the gels was detected as described above. The specimens that showed a differential band at SSCP were amplified to obtain templates for DNA sequencing. These amplifications were independent of those used for SSCP analysis. Amplified DNA fragments were purified from 0.9% agarose gels using the Geneclean kit (Bio-101 Inc., La Jolla, CA) and sequenced using an ABI Prim 377 DNA apparatus (PE Applied Biosystems).

Mutational Study of the K-*ras* Oncogene. PCR amplification of the K-*ras* gene, using 50 ng of DNA as template, was carried out in a 10 μl reaction volume with a final concentration of 1 × PCR buffer and 0.2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc.), 200 μM dNTP mix, 0.6 of each primer, 2.5 mM $MgCl_2$, and distilled H_2O to reach the total volumes. Each sample was denatured at 94°C for 5 min and subjected to 30 cycles (94°C for 30 s, 58°C for 40 s, and 72°C for 30 s), followed by a final 7-min extension at 72°C. The amplified products were mixed with 10 μl of denaturing stop solution (see "Mutational study of the *p53* gene"), heated to 95°C for 5 min, and rapidly cooled on ice. The samples were electrophoresed on nondenaturing 12% polyacrylamide gels at 250 v overnight at 4°C. Products were visualized by nonradioisotopic means. The primers used for amplification of exon 1 of K-*ras*, which contains codons 12 and 13, were: 5′GACTGAATATAAACTTGTGGTAGT and 5′CTATTGTTGGATCATATTCGTCC. All of the specimens showing a differential band at SSCP were sequenced following the method used for the *p53* gene.

Promoter Hypermethylation. p16INK4a and p14ARF aberrant promoter methylation was analyzed in tumor, mononuclear blood cells, and plasma DNA. DNA methylation patterns in the CpG island of the *p16INK4a* and *p14ARF* genes were determined by methylation-specific PCR as described elsewhere (41). Briefly, 1 μg of DNA from tumor, blood cells, and plasma was denatured by NaOH, and subsequently treated with

[3] The abbreviations used are: TUR, transurethral resection; dNTP, deoxynucleotide triphosphate; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.

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hydroquinone and sodium bisulfite at 53°C for 16 h. Modified DNA samples were purified using the Wizard DNA purification resin following the manufacturer instructions (Promega) and eluted into 50 μl of water. NaOH was added to complete the modification followed by ethanol precipitation. Resuspended DNA was used in a PCR reaction. Primer sequences and PCR conditions for *p16INK4a* and *p14ARF* are reported elsewhere (41, 42). Placental DNA treated with *SssI* methyltransferase and DNA of Raji, KMH2, and L-540 cell lines were used as positive controls for methylated alleles. DNA from normal lymphocytes was used as a negative control for methylated genes. Samples were electrophoresed on nondenaturing 8% gels at 450 v for 2–3 h and visualized by nonradioisotopic techniques.

Statistical Analysis. The variables were contrasted using the χ^2 test. Differences are considered significant where $P < 0.05$. Statistical analyses were performed using the EPI-INFO package, version 6.04.

Results

Microsatellite Analysis. Fifteen bladder carcinoma patients (56%) showed allelic loss in at least one locus. Nine of these (60%) had the same microsatellite alteration in their plasma DNA. The marker D9S161 showed the highest individual rate of LOH in tumor DNA (30%), but D9S747 showed the highest correlation between tumor and plasma DNA (100%; Fig. 1*A*). In 6 cases, plasma DNA revealed an additional alteration, which was not present in tumor DNA (Fig. 1*B*), although 3 of these cases showed LOH in their tumor and plasma samples for some of the other microsatellite markers.

***p53* Gene Mutations.** Only 1 tumor patient showed a mutation in exon 8 of *p53*, which was not found in the corresponding plasma sample (Fig. 1*C*). Another patient presented a nucleotide change in exon 6 of *p53* in both normal blood cells and tumor DNA, but again, it was not detected in plasma. The incidence of *p53* mutations was lower (3.7%) than in other studies (43, 44), but in a recent report, 1% of *p53* mutations were detected by microarray technology (45).

K-*ras* Gene Mutations. In 2 cases, the same K-*ras* mutations were identified in both tumor tissue and plasma (Fig. 1*D*). One case showed *de novo* K-*ras* mutation in plasma, which was not detected in its tumor counterpart. This case showed LOH for D9S747 and D13S310 microsatellite markers in the tumor and plasma samples. Mutations in the K-*ras* oncogene are rare in human bladder cancers (46).

Methylation Status of the *p14ARF* and *p16INK4a* Promoters. Methylation of 5′ regulatory regions of CG dinucleotides, called CpG islands, is a well-established mechanism of transcriptional repression (47). *p14ARF* and *p16INK4a* are candidates for hypermethylation-associated inactivation, because they contain documented CpG islands that can be silenced by this epigenetic alteration in many tumor types (48–50). Of the bladder tumor samples amplified, 15 (56%) and 5 (18%) presented *p14ARF* and *p16INK4a* promoter hypermethylation, respectively. Of these, 13 (87%) and 2 (40%) showed *p14ARF* and *p16INK4a* promoter hypermethylation in plasma samples, respectively (Fig. 1, *E* and *F*). All of the cases with *p16INK4a* promoter hypermethylation were positive for *p14ARF* promoter hypermethylation. Bisulfite-modified DNA from the 27 normal blood cells samples was not amplified with either primers. To our knowledge, *p14ARF* promoter hypermethylation has not been analyzed in bladder cancer patients and we cannot compare our results. However, the percentage found for *p16INK4a* promoter hypermethylation is within reported ranges for bladder carcinomas (51, 52).

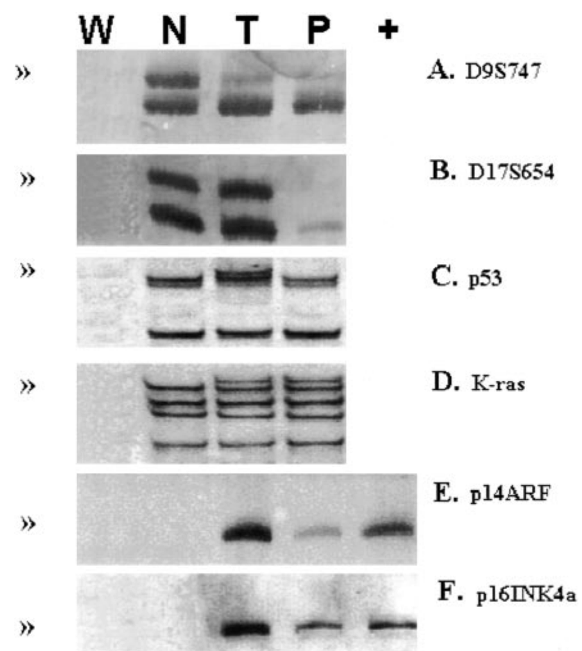

Fig. 1 Photograph showing: *A,* LOH in the D9S747 microsatellite in tumor and plasma DNA. *B,* LOH in the D17S654 marker in plasma DNA but not found in tumor DNA. *C,* p53 mutation in tumor but not in plasma DNA. *D,* K-ras mutation in both tumor and plasma DNA. *E,* p14ARF promoter hypermethylation in tumor and plasma DNA. *F,* p16INK4a promoter hypermethylation in tumor and plasma DNA. *W,* water blanks as negative controls; *N,* normal mononuclear blood cells DNA; *T,* tumor DNA; *P,* plasma DNA; +, positive controls, KMH2 cell line for p14ARF methylation and Raji cell line for p16INK4a methylation.

Correlations between Clinicopathological Parameters and Molecular Changes. Of the 27 patients, 21 (78%) showed in the tumor sample at least one of the alterations analyzed. A significant correlation was found between tumor p14ARF promoter hypermethylation and multicentric foci ($P = 0.04$), tumor size ($P = 0.006$), bladder muscle invasion ($P = 0.01$), and stage ($P = 0.01$). Nearly statistically significant was lymph nodes enlargement ($P = 0.05$; Table 1). No statistical associations were found in regard to LOH in the tumor samples.

Twenty cases (74%) showed at least one of the alterations in plasma DNA (17 cases displayed the same alterations in their tumor and plasma samples, and 3 cases showed LOH in plasma but not in their tumor samples for any of the microsatellites analyzed). There was a significant association between p14ARF hypermethylation in plasma DNA and multicentric foci ($P = 0.03$), larger tumors ($P = 0.01$), and relapse ($P = 0.03$). Close to statistical significance ($P = 0.05$) were lymph node enlargement (the only 3 patients harboring positive nodes showed promoter hypermethylation), invasion of the bladder muscle, more advanced stages, and the treatment applied; the 3 patients

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Table 1 Methylation status of bladder tumors

Statistical associations between bladder cancer cases with tumor DNA p14ARF promoter hypermethylation and tumor samples that do not show this alteration in regard to various clinicopathological parameters.

	Tumor *p14ARF* methyl		Tumor *p14ARF* unmethyl		
	n[a]	%	*n*	%	*P*
No. of patients	15		12		
Multicentric foci					
Yes	15	100	9	75	0.04
No	0	0	3	25	
Tumor size					
≤3	0	0	5	42	0.006
>3	15	100	7	58	
Invasion					
T_a–T_1	9	60	12	100	0.01
T_2–T_4	6	40	0	0	
Stage					
0_a–II	9	60	12	100	0.01
III-IV	6	40	0	0	
LNE					
Positive	4	27	0	100	0.05
Negative	11	73	12	0	

[a] *n*, number of cases; LNE, lymph node enlargement.

subjected to more aggressive therapy, *i.e.,* cystectomy, showed p14ARF promoter hypermethylation in their plasma DNA (Table 2). A statistical association between plasma LOH in at least one of the markers analyzed and recurrence of the disease was detected ($P = 0.02$; Table 2).

The association with the clinical parameters was not analyzed for p53 and K-ras mutations, and p16INK4a aberrant methylation because of the very low number of cases showing these alterations, which could lead to misinterpretation results.

Discussion

This is the first study to identify a possible indicator of disease recurrence for bladder cancer in plasma DNA.

Twenty-six transitional cell carcinomas and 1 squamous tumor, and their corresponding mononuclear blood cells and plasma samples were analyzed for DNA microsatellite alterations, *p53* and *K-ras* mutations, and *p14ARF/p16INK4a* promoter hypermethylation. Seventeen cases showed the same alteration in plasma and tumor DNA, indicating that this plasma DNA derives from the primary tumor. Six cases presented a new alteration in plasma, which was not found in the tumor counterpart. Most studies on plasma DNA reveal a few alterations in plasma only, mainly when microsatellite polymorphic markers are used to characterize tumor and plasma DNA (22, 26). Some authors suggest that plasma alterations could be artifacts (27, 28), although they are not detected in healthy controls (26, 35). Other authors point to a heterogeneous clonal origin (22, 24). The common multiple foci found in bladder cancer patients may have a monoclonal origin, which is followed by cellular spreading (8–10). Once the microfoci have migrated, they may evolve and acquire new alterations. In this case, the specific alterations in plasma DNA but not in the tumor sample could be explained by the fact that this DNA proceeds from other tumor foci which harbor the alteration, as we have described recently for colon carcinomas (53).

Table 2 Methylation status of tumor plasma DNA

Statistical associations between bladder cancer cases with DNA plasma alterations (*p14ARF* promoter hypermethylation or microsatellite changes) and cases with no alterations regarding various clinicopathological parameters.

	Plasma *p14ARF* methyl		No plasma *p14ARF* methyl		
	n[a]	%	*n*	%	*P*
No. of patients	13		14		
Multicentric foci					
Yes	13	100	10	71	0.036
No	0	0	4	29	
Tumor Size					
≤3	0	0	5	36	0.01
>3	13	100	9	64	
Relapse					
Yes	9	69	4	29	0.03
No	4	31	10	71	
LNE					
Positive	3	23	0	0	0.05
Negative	10	77	14	100	
Invasion					
T_a–T_1	8	62	13	93	0.05
T_2–T_4	5	38	1	7	
Treatment					
TUR/IT	10	77	14	100	0.05
Cystectomy	3	23	0	0	
Stage					
0_a–II	8	62	13	93	0.05
III–IV	5	38	1	7	

Allelic losses in tumor plasma DNA

	Plasma LOH		No plasma LOH		
	n	%	*n*	%	*P*
No. of patients	16		11		
Relapse					
Yes	10	62.5	2	18	0.02
No	6	37.5	9	82	

[a] *n*, number of cases; LNE, lymph node enlargement; IT, intravesical therapy.

The low proportion of *p53* and *K-ras* mutations, and *p16INK4a* hypermethylation in our series of bladder cancer samples indicates that they are not suitable markers. Nevertheless, microsatellite alterations or *p14ARF* promoter hypermethylation was detected in 56% of tumors, but concordance between tumor and plasma alterations was higher for *p14ARF* status (87% *versus* 60%). *p14ARF* promoter hypermethylation has been reported in other tumors, such as colorectal (42), gastric (54), breast (55), ovarian (56), oral squamous cell carcinomas (57), oligodendrogliomas (58), glioblastomas (59), and central nervous system lymphomas (60).

It is remarkable that recurrence was associated with p14ARF promoter hypermethylation or LOH only when the alteration was present in plasma. This observation could be because of the specificity to find alterations in the plasma sample. Because of the multicentric foci in bladder cancer, we could have a tumoral sample from a focus not showing the alteration observed in the plasma. Thus, the case would be classified inaccurately as without alteration, which could modify the results.

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Although alterations in plasma, LOH, and *p14ARF* promoter aberrant methylation could predict relapse, *p14ARF* promoter hypermethylation is a better prognosis marker for several reasons. Firstly, although in our subset of bladder cancer patients the proportion of these alterations in the tumors was identical (56%), concordance between presence in tumor and plasma DNA was higher for *p14ARF* aberrant promoter methylation. Secondly, for microsatellite analysis, some of these markers should be tested to obtain an acceptable rate of LOH. To check the promoter status of a gene, only one reaction is required.

Improvements in molecular and genetic approaches have led to the identification of tumor-derived nucleic acids in the plasma of cancer patients (20–22, 24, 26, 27, 31, 32, 37, 61, 62), although the mechanisms by which these nucleic acids are released into plasma remain unknown (62–65). It is suggested that the alterations found in the plasma DNA of these patients could be used as a prognostic factor. We have detected plasma DNA with tumor features in a series of 27 bladder cancer patients. *p14ARF* promoter hypermethylation may be a useful prognostic factor, because it could be associated with recurrence disease and could be clinically relevant as a noninvasive tool for the detection of relapses during the follow-up of patients.

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References

1. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics 1996. CA Cancer J. Clin., *46:* 5–27, 1996.

2. Zang, Z. F., Sarkis, A. S., Cordón-Cardó, C., Dalbagni, G., Melamed, J., Aprikian, A., Pollack, D., Shienfeld, J., and Herr, H. W. Tobacco smoking, occupation, and p53 overexpression in early stage bladder cancer. Cancer Epidemiol. Biomark. Prev., *3:* 19–24, 1994.

3. Fair, W. R., Fuks, Z. Y., and Scher, H. I. Cancer of the bladder. *In:* V. DeVita, Jr., S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles & Practice of Oncology, pp.1052–1072. Philadelphia: JB. Lippincott. 1994.

4. Wolf, H., Kakizoe, T., Smith, P. H., Brosman, S. A., Okajima, E., Rubben, H., and Utz, D. C. Bladder tumors. Treated natural history. Prog. Clin. Biol. Res., *221:* 223–255, 1986.

5. Raghavan, D., Shipley, W., Garnick, M., Russel, P., and Richie, J. Biology and management of bladder cancer. N. Engl. J. Med., *322:* 1129–1138, 1990.

6. Catalona, W. J. Urothelial tumors of the urinary tract. *In:* P. C. Walsh, A. B. Retik, T. A. Stanley, and E. D. Vaughan, Jr. (eds.), Campbell's Urology, pp. 1094–1158. Philadelphia: WB Saunders Co., 1992.

7. Whitmore, W. F., Jr. Toward the rational management of bladder cancer: an overview. Urology, *31:* 5–8, 1998.

8. Sidransky, D., Frost, P., von Eschenbach, A., Oyasu, R., Preisinger, A. C., and Vogelstein, B. Clonal origin of bladder cancer. N. Engl. J. Med., *326:* 737–740, 1992.

9. Mao, L., Lee, D. J., Tockman, M. S., Erozan, Y. S., Askin, F., and Sidransky, D. Microsatellite alterations as clonal markers for the detection of human cancers. Proc. Natl. Acad. Sci. USA, *91:* 9871–9875, 1994.

10. Habuchi, T., Takahashi, R., Yamada, H., Kakehi, Y., Sugiyama, T., and Yoshida, O. Metachronous multifocal development of urothelial cancers by intraluminal seeding. Lancet, *342:* 1087–1088, 1993.

11. Richter, J., Beffa, L., Wagner, U., Schrmal, P., Gasser, T., Moch, H., Mihatsch, M., and Sauter, G. Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. Am. J. Pathol., *153:* 1615–1621, 1998.

12. Simon, R., Bürger, H., Brinkschmidt, C., Böcker, W., Hertle, L., and Terpe, H. Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. J. Pathol., *185:* 345–351, 1998.

13. Voorter, C., Joos, S., Bringuier, P. P., Vallinga, M., Poddighe, P., Schalken, J., du Manoir, S., Ramaekers, F., Lichter, P., and Hopman, A. Detection of chromosomal imbalances in transitional cell carcinoma of the bladder by comparative genomic hybridization. Am. J. Pathol., *146:* 1341–1354, 1995.

14. Knowles, M. A., Elder, P. A., Williamson, M., Cairns, J. P., Shaw, M. E., and Law, M. G. Allelotype of human bladder cancer. Cancer Res., *54:* 531–538, 1994.

15. Kallioniemi, A., Kallioniemi, O., Citro, G., Sauter, G., DeVries, S., Kerschmann, R., Caroll, P., and Waldman, F. Identification of novel DNA amplifications and deletions in primary bladder cancer by comparative genomic hybridization. Gene Chromosome Cancer, *12:* 213–219, 1995.

16. Shapiro, B., Chakrabarty, M., Cohn, E. M., and Leon, S. A. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. Cancer (Phila.), *51:* 2116–2120, 1983.

17. Leon, S. A., Shapiro, B., Sklaroff, D. M., and Yaros, J. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res., *37:* 646–650, 1997.

18. Mulcahy, H. E., Lyautey, J., Lederrey, C., Cheng, X. Q., Anker, P., Alstead, E. M., Ballinger, A., Farthing, M. J., and Stroun, M. A prospective study of K-ras mutations in the plasma of pancreatic cancer patients. Clin. Cancer Res., *4:* 271–275, 1998.

19. Vasioukhin, V., Anker, P., Maurice, P., Lyautey, J., Lederrey, C., and Stroun, M. Point mutation of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br. J. Haematol., *86:* 774–779, 1994.

20. Silva, J. M., Gonzalez, R., Dominguez, G., Garcia, J. M., España, P., and Bonilla, F. TP53 gene mutations in plasma DNA of cancer patients. Gene Chromosome Cancer, *24:* 160–161, 1999.

21. Nawroz, H., Koch, W., Anker, P., Stroun, M., and Sidransky, D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat. Med., *2:* 1035–1037, 1996.

22. Goessl, C., Heicapell, R., Münker, R., Anker, P., Stroun, M., Krause, H., Müllr, M., and Miller, K. Microsatellite analysis of plasma DNA from patients with clear cell renal carcinoma. Cancer Res., *58:* 4728–4732, 1998.

23. Wong, I. H. N., Lo, D., Zhang, J., Liew, C. T., Ng, L., Wong, N., Lai, S., Lau, Y., Hjelm, M., and Johnson, P. J. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res., *59:* 71–73, 1999.

24. Chen, X. Q., Stroun, M., Magnenat, J. L., Nicod, L. P., Kurt, A. M., Lyautey, J., Lederrey, C., and Anker, P. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat. Med., *2:* 1033–1035, 1996.

25. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. Cancer Res., *59:* 67–70. 1999.

26. Silva, J. M., Dominguez, G., Garcia, J. M., Gonzalez, R., Villanueva, M. J., Navarro, F., Provencio, M., San Martin, S., España, P., and Bonilla, F. Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res., *59:* 3251–3256, 1999.

27. Chen, X., Bonnefoi, H., Diebol-Berger, S., Lyautey, J., Lederrey, C., Faltin-Traub, E., Stroun, M., and Anker, P. Detecting of tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin. Cancer Res., *5:* 2297–2303, 1999.

28. Mayall, F., Fairweather, S., Wilkins, R., Chang, B., and Nicholls, R. Microsatellite abnormalities in plasma of patients with breast carcinoma: concordance with the primary tumor. J. Clin. Pathol., *52:* 363–366, 1999.

29. Anker, P., Lefort, F., Vasioukhin, V., Lyautey, J., Lederrey, C., Chen, X. Q., Stroun, M., Mulcahy, H. E., and Farthing, M. J. K-ras mutations are found in DNA extracted from the plasma of patients with colorectal cancer. Gastroenterology, *112:* 1114–1120, 1997.

30. Castells, A., Puig, P., Mora, J., Boadas, J., Boix, L., Urgell, E., Sole, M., Capella, G., Lluis, F., Fernandez-Cruz, L., Navarro, S., and Farre, A.

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K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. J. Clin. Oncol., *17:* 578–584, 1999.

31. Sozzi, G., Conte, D., Mariani, L., Lo Vullo, S., Roz, L., Lombardo, C., Pierotti, M., and Tavecchio, L. Analysis of circulating tumor DNA in plasma at diagnosis and during follow-up of lung cancer patients. Cancer Res., *61:* 4675–4678, 2001.

32. Taback, B., Fujiwara, Y., Wang, H-J., Foshag, L. J., Morton, D. L., and Hoon, D. S. B. Prognostic significance of circulating microsatellite markers in the plasma of melanoma patients. Cancer Res., *61:* 5723–5726, 2001.

33. Kopreski, M. S., Benko, F. A., Borys, D. J., Khan, A., McGarrity, T. J., and Gocke, C. D. Somatic mutations screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. J. Natl. Cancer Inst., *92:* 918–923, 2000.

34. Utting, M., Werner, W., Muller, G., Schubert, J., and Junker, K. A possible noninvasive method for the detection of bladder cancer in patients: microsatellite analysis of free DNA in urine and blood. Ann N. Y. Acad Sci., *945:* 31–35, 2001.

35. Von Knobloch, R., Hegele, A., Brandt, H., Olbert, P., Heidenreich, A., and Hofmann, R. Serum DNA and urine DNA alterations of urinary transitional cell bladder carcinoma detected by fluorescent microsatellite analysis. Int. J. Cancer, *94:* 67–72, 2001.

36. Gerken, S. C., Albertsen, H., Elsner, T., Ballard, L., Holik, P., Lawrence, E., Moore, M., Zhao, X., and White, R. A strategy for constructing high-resolution genetic maps of the human genome: a genetic map of chromosome 17p, ordered with meiotic breakpoint-mapping panels. Am. J. Hum. Genet., *56:* 484–499, 1995.

37. Steiner, G., Schoenberg, M. P., Linn, J. F., Mao, L., and Sidransky, D. Detection of bladder cancer recurrence by microsatellite analysis of urine. Nat. Med., *3:* 621–624, 1997.

38. Silva, J. M., Gonzalez, R., Provencio, M., Dominguez, G., Garcia, J. M., Gallego, I., Palacios, J., España, P., and Bonilla, F. Loss of heterozygosity in BRCA1 and BRCA2 markers and high-grade malignancy in breast cancer. Breast Cancer Res. Treat., *53:* 9–17, 1999.

39. Deng, G., Lu, Y., Zlotnikov, G., Thor, A. N., and Smith, H. S. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science (Wash. DC), *274:* 2057–2059, 1996.

40. Brenner, A. J., and Aldaz, C. M. Chromosome 9p allelic loss and p16/CDKN2 in breast cancer and evidence of p16 inactivation in immortal breast epithelial cells. Cancer Res., *55:* 2892–2895, 1995.

41. Herman, J. G., Graff, J. R., Myöhänen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR. a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

42. Esteller, M., Tortola, S., Toyota, M., Capella, G., Peinado, M. A., Baylin, S. B., and Herman, J. G. Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and p53 mutational status. Cancer Res., *60:* 129–133, 2000.

43. Masuda, A., Kikuchi, Y. Y., and Kawamura, N. Mutational analysis of the p53 tumor suppressor gene using paraffin-embedded specimens of human transitional cell carcinomas by the direct sequencing method. Tokai J. Exp. Clin. Med., *25:* 69–77, 2000.

44. Berggren, P., Steineck, G., Adolfsson, J., Hansson, J., Jansson, O., Larsson, O., Larsson, P., Sandstedt, B., Wijkstrom, H., and Hemminki, K. p53 mutations in urinary bladder cancer. Br. J. Cancer., *84:* 1505–1511, 2001.

45. Wikman, F. P., Lu, M. L., Thykjaer, T., Olesen, S. H., Andersen, L. D., Cordon-Cardo, C., Ornto, T. J. Evaluation of the performance of a p53 sequencing microarray chip using 140 previously sequenced bladder tumor samples. Clin Chem., *46:* 1555–1561, 2000.

46. Przybojewska, B., Jagiello, A., and Jalmuzna, P. H-ras. K-ras and N-ras gene activation in human bladder cancers. Cancer Genet. Cytogenet., *121:* 73–77, 2000.

47. Bird, A. The essential of the DNA methylation. Cell, *70:* 5–8, 1992.

48. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones, P. A. Methylation of the 5′ CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.

49. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor CDKN2/p16 in human cancers. Nat. Med., *7:* 686–692, 1995.

50. Robertson, K. D., and Jones, P. A. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and downregulated by wildtype p53. Mol. Cell Biol., *18:* 6457–6473, 1998.ç.

51. Tsutsumi, M., Tsai, Y. C., Gonzalgo, M. L., Nichols, P. W., and Jones, P. A. Early acquisition of homozygous deletions of p16/p19 during squamous cell carcinogenesis and genetic mosaicism in bladder cancer. Oncogene, *17:* 3021–3027, 1998.

52. Orlow, I., LaRue, H., Osman, I., Lacombe, L., Moore, L., Rabbani, F., Meyer, F., Fradet, Y., and Cordon-Cardo, C. Deletions of the INK4A gene in superficial bladder tumors. Association with recurrence. Am. J. Pathol., *155:* 105–113, 1999.

53. Garcia, J. M., Silva, J. M., Dominguez, G., Silva, J., and Bonilla, F. Heterogeneous tumor clones as an explanation of discordance between plasma DNA and tumor DNA alterations. Genes Chromosome Cancer, *31:* 300–301, 2001.

54. Iida, S., Akiyama, Y., Nakajima, T., Ichikawa, W., Nihei, Z., Sugihara, K., and Yuasa, Y. Alterations and hypermethylation of the p14(ARF) gene in gastric cancer. Int. J. Cancer, *87:* 654–658, 2000.

55. Silva, J., Dominguez, G., Silva, J. M., Garcia, J. M., Gallego, I., Corbacho, C., Provencio, M., España, P., and Bonilla, F. Analysis of genetic and epigenetic processes that influence p14ARF expression in breast cancer. Oncogene, *20:* 4586–4590, 2001.

56. Hashiguchi, Y., Tsuda, H., Yamamoto, K., Inoue, T., Ishiko, O., and Ogita, S. Combined analysis of p53 and RB pathways in epithelial ovarian cancer. Hum. Pathol., *32:* 988–996, 2001.

57. Shintani, S., Nakahara, Y., Mihara, M., Ueyama, Y., and Matsumura, T. Inactivation of the p14(ARF), p15(INK4B) and p16(INK4A) genes is a frequent event in human oral squamous cell carcinomas. Oral Oncol., *37:* 498–504, 2001.

58. Watanabe, T., Nakamura, M., Yonekawa, Y., Kleihues, P., and Ohgaki, H. Promoter hypermethylation and homozygous deletion of the p14ARF and p16INK4a genes in oligodendrogliomas. Acta Neuropathol., *101:* 185–189, 2000.

59. Nakamura, M., Watanabe, T., Klangby, U., Asker, C., Wiman, K., Yonekawa, Y., Kleihues, P., and Ohgaki, H. p14ARF deletion and methylation in genetic pathways to glioblastomas. Brain Pathol., *11:* 159–168, 2001.

60. Nakamura, M., Sakaki, T., Hashimoto, H., Nakase, H., Ishida, E., Shimada, K., and Konishi, N. Frequent alterations of the p14(ARF) and p16(INK4a) genes in primary central nervous system lymphomas. Cancer Res., *61:* 6335–6339, 2001.

61. Fujiwara, Y., Chi, D. D., Wang, H., Keleman, P., Morton, D. L., Turner, R., and Honn, D. S. Plasma DNA microsatellite as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res., *59:* 1567–1571, 1999.

62. Lo, Y. M., Chan, L. Y., Lo, K. W., Leung, S. F., Zhang, J., Chan, A. T., Lee, J. C., Hjelm, N. H., Johnson, P. S., and Huang, D. P. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. Cancer Res., *59:* 1188–1191, 1999.

63. Fournie, G. J., Courtin, J. P., Laval, F., Chale, J. J., Pourrat, J. P., Pujazon, M. C., Lauque, D., and Carles, P. Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumors. Cancer Lett., *91:* 221–227, 1995.

64. Rogers, J. C., Boldt, D., Kornfeld, S., Skinner, A., and Valeri, C. R. Excretion of deoxyribonucleic acid by lymphocytes stimulated with phytohemagglutinin or antigen. Proc. Natl. Acad. Sci. USA, *69:* 1685–1689, 1972.

65. Anker, P., Mulcahy, H., Chen, X. Q., and Stroun, M. Detection of circulating tumor DNA in the blood (plasma/serum) of cancer patients. Cancer Metastasis Rev., *18:* 65–73, 1999.

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