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11. Eyes from macaque (*Macaca nemestrina*, *Macaca fascicularis*, or *Macaca mulatta*) were enucleated after euthanasia. The retina, together with the pigment epithelium and choroid layer, was dissected and mounted in a superfusion chamber on the stage of a light microscope. Temperature was maintained in the chamber at 36° ± 1°C and superfused at ~5 ml/min. Vital labeling of horizontal cell nuclei was achieved by incubating the eye cup before retinal dissection for ~20 min in Ames medium to which diamidinophenylindole (DAPI) was added at a concentration of ~10 μM. Intracellular penetrations were made under direct visual control with high-resistance micropipettes, and light responses were recorded in conventional bridge mode. The recorded cells were directly observed during recording by iontophoretic injection of the fluorescent dye pyranine. At the termination of recording, cells were preserved for later analysis by injection of Neurobiotin and subsequent horseradish peroxidase histochemistry after tissue fixation (*10*).
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16. Output of the LEDs was modulated by a circuit driver under computer control (*23*). A spectroradiometric calibration was performed in the plane of the retina with a Gamma Scientific spectral radiometer. Radiometric measures were converted to photometric units (illuminance) by convolution with the human photopic spectral luminosity function (*24*). We estimated retinal illuminance in these experiments to be ~1000 trolands.
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18. The relative LED modulations required for L-, M-, or S-cone isolation were calculated from the Smith-Pokorny cone fundamentals (*25*). Cone contrast was expressed as Michelson contrast $C = (Q_{max} - Q_{min})/(Q_{max} + Q_{min})$, where Q_{max} and Q_{min} correspond to the quantal absorptions calculated from the cone fundamentals. We confirmed S-cone isolation by checking for a null response in M-parasol ganglion cells, which were previously shown to lack S-cone input (*26*).
19. No other horizontal cell response properties or distinctive dendritic morphologies were found, despite our large sample of recorded cells. All of the morphological features attributed to a third (H3) horizontal cell type (*9*) were encompassed in our sample of H1 cells; H3 cells appear to reflect normal variation in the H1 cell population, with no separate physiological role. Our picture of the H1 and H2 cell mosaic is consistent with previous evidence that single cells can either avoid or "seek out" S-cones (*9*). This conclusion was recently confirmed by combining intracellular labeling of the horizontal cells and staining of the S-cone pedicles with an antibody to S-cone opsin [A. C. Goodchild, T. L. Chan, U. Grunert, *Soc. Neurosci. Abstr.* **21**, 1645 (1995)].
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Molecular Detection of Primary Bladder Cancer by Microsatellite Analysis

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Microsatellite DNA markers have been widely used as a tool for the detection of loss of heterozygosity and genomic instability in primary tumors. In a blinded study, urine samples from 25 patients with suspicious bladder lesions that had been identified cystoscopically were analyzed by this molecular method and by conventional cytology. Microsatellite changes matching those in the tumor were detected in the urine sediment of 19 of the 20 patients (95 percent) who were diagnosed with bladder cancer, whereas urine cytology detected cancer cells in 9 of 18 (50 percent) of the samples. These results suggest that microsatellite analysis, which in principle can be performed at about one-third the cost of cytology, may be a useful addition to current screening methods for detecting bladder cancer.

There are approximately 50,000 new cases of bladder cancer each year in the United States; it is the fourth most common cancer in men and the eighth most common in women (*1*). Urine cytology is a common noninvasive procedure for the diagnosis of this disease (*2*), but it can miss up to 50% of tumors (*3*). The "gold standard" for diagnosis is cystoscopy, which allows visualization and direct biopsy of suspicious bladder lesions. However, because cystoscopy is an expensive and invasive procedure, it cannot be used as a general screening tool for the detection of bladder cancer. Because early diagnosis of bladder cancer is critical for successful treatment, there is a pressing need for more sensitive and cost-effective diagnostic tools (*4*).

Multiple genetic changes occur during the development of primary bladder cancer (*5*). For example, mutations in the tumor suppressor gene p53 signal the progression to invasiveness and have been successfully used as molecular markers for the detection of cancer cells in urine samples (*6*). However, this diagnostic strategy has limited clinical application because the techniques are cumbersome and because p53 mutations appear relatively late in the disease (*6*, *7*).

We have suggested that microsatellite markers might be useful as clonal markers for the detection of human cancer (*8*) because simple DNA repeat alterations can be readily detected in clinical samples by the polymerase chain reaction (PCR). Here, we tested this suggestion in a pilot study of bladder cancer patients. We first screened 60 trinucleotide and tetranucleotide markers in the DNA from 50 anonymous primary bladder cancers (*9*). Of these cancers, 40 (80%) contained at least one marker alteration when compared with the DNA from matched normal lymphocytes. We calculated that a panel of the 10 most useful markers would theoretically detect alterations in 52% of all cancers (*10*).

Twenty-five patients presented with symptoms suggestive of bladder cancer (for example, gross hematuria) and were found to harbor suspicious lesions at cystoscopy. Urine samples were collected (before cys-

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toscopy) and were then distributed in blinded fashion for microsatellite analysis and routine urine cytology. We then tested the DNA in the urine sediment from these 25 patients with suspicious bladder lesions and from five controls (patients without evidence of bladder cancer) (Table 1). Urine and lymphocyte DNA from each patient were amplified by PCR, and polymorphic alleles were compared at the 10 preselected microsatellite loci. The urine DNA of 10 patients contained a microsatellite alteration (expansion or deletion of a repeat unit), in close agreement (after the code was broken; see below) with the frequency expected on the basis of our calculations (*10*) (Table 1).

In addition to microsatellite alterations, primary tumors often harbor chromosomal deletions at suppressor gene loci that are manifested as loss of heterozygosity (LOH) and are readily detected by microsatellite analysis. Notably, 18 urine DNA samples also demonstrated LOH, particularly with marker D9S747 from chromosome 9p21 (Fig. 1). This result is consistent with our observation that loss of chromosome 9 occurs frequently in bladder cancer (*11*). Analysis of three additional dinucleotide markers on chromosome 9p21 (D9S171, D9S162, and IFNA) for LOH confirmed the presence of deletions in urine samples

Table 1. Characteristics of patients with bladder lesions. Cyto, cytology; Alt, microsatellite alteration (expansion or deletion); ND, not done; NA, not applicable; TCC, transitional cell carcinoma; AC, adenocarcinoma; mucin, mucinous; inflamm, inflammation. Grades and stages were assigned following the recommendations of the American Joint Committee on Cancer after examination by pathology (*20*). Control patients presented without signs or symptoms of bladder cancer (for example, benign prostatic enlargement). Four of the five control patients underwent cystoscopy and were free of any suspicious lesions.

Patient	Age/sex	Tumor grade	Pathology	Stage	Urine analysis		
					Cyto	LOH	Alt
1	80/F	G3	TCC	T3N0M0	+	+	−
2	43/M	ND	TCC		ND	+	−
3	75/M	G3	TCC	T1N0M0	−	−	+
4	60/M	G1	TCC in situ	TaN0M0	−	+	−
5	78/F	G2	TCC		+	+	+
6	45/M	G1	TCC	T1N0M0	Atypia	−	+
7	72/M	*	AC + mucin		+	+	−
8	84/M	ND	TCC	T1N0M0	+	+	+
9	75/M	G3	TCC	T1N0M0	+	−	−
10	82/F	G3	TCC	T4N2M0	Atypia	+	−
11	80/M	G3	TCC in situ	TaN0M0	Atypia	+	+
12	67/M	G2	TCC	T1N0M0	ND	+	−
13	75/M	G3	TCC	T2N0M0	−	+	+
14	86/F	G3	TCC in situ	TaN0M0	−	+	−
15	71/M	G2	TCC		−	+	−
16	54/M	G3	TCC	T3N0M0	+	+	+
17	51/M	G2	TCC	T1N0M0	+	+	−
18	72/M	*	Intraductal TCC		+	+	+
19	65/M	G2	TCC	T2N0M0	+	+	−
20	55/F	G1–G2	TCC	TaN0M0	−	−	+
21	65/F	NA	Atypia/inflamm		ND	+	+
22	86/M	NA	Chronic inflamm		ND	−	−
23	71/M	NA	Chronic inflamm		−	−	−
24	79/M	NA	Atypia/inflamm		Atypia	+	−
25	65/M	NA	Normal		−	−	−
26	70/M	Control patient without cancer		NA	ND	−	−
27	70/M	Control patient without cancer		NA	ND	−	−
28	54/M	Control patient without cancer		NA	ND	−	−
29	35/M	Control patient without cancer		NA	ND	−	−
30	68/M	Control patient without cancer		NA	ND	−	−

*Prostatic fossa.

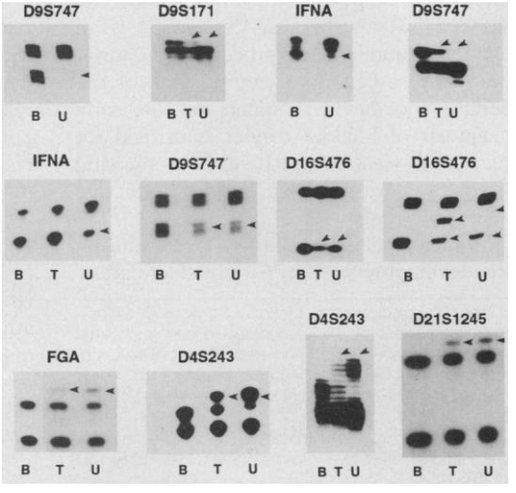

Fig. 1. Microsatellite analysis of urine sediment. Blood (B), urine (U), and tumor (T) DNA was isolated and amplified by PCR (*9*). Polymorphic microsatellite markers are designated above each block. LOH in urine DNA is shown in patients 11, 5, 1, and 5 (top row, left to right) and in patients 17, 16, 7, and 1 (middle row, left to right). Arrowheads denote loss of the allele in the urine and in the corresponding tumor sample. Patient 1 (middle row, far right) demonstrates LOH in the tumor and urine, but an alteration (smaller allele migrating just below the top allele) appears only in the tumor (see Table 2). Microsatellite alterations in urine DNA and in the corresponding tumor are shown in patients 20, 5, 20, and 16 (bottom row, left to right). In this row, arrowheads denote altered alleles (expanded or deleted repeats). The allele sizes of these markers (available from the authors) varied in each patient. In some cases, the alteration or LOH was more pronounced in the urine DNA than in the tumor DNA (or was present only in the urine DNA) because only a fraction of the tumor biopsy was composed of neoplastic cells.

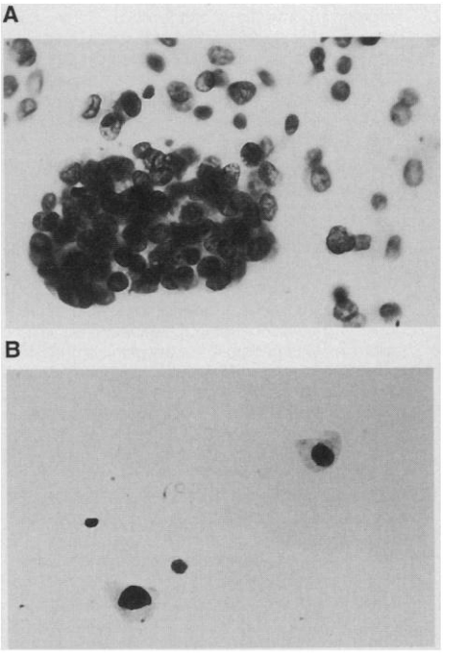

Fig. 2. Cytology of urine sediment. (**A**) Transitional cell carcinoma. Tumor cells with enlarged hyperchromatic nuclei and a high nucleus/cytoplasm ratio (patient 8, Papanicolaou stain) are shown. (**B**) Atypical transitional cells. Two atypical cells with enlarged hyperchromatic nuclei and a low nucleus/cytoplasm ratio (patient 10) are shown. Both nuclei and cytoplasm show degenerative changes that could be the cause of the hyperchromasia and irregularities of nuclear contour. Although a transitional cell carcinoma is suspected in this case, a definitive diagnosis cannot be made (Papanicolaou stain). Both (A) and (B) were correctly identified as cancer cells by molecular analysis of urine DNA.

that demonstrated loss of chromosome 9 with marker D9S747 (Table 2, patients 5, 8, and 16).

When the code was broken, we learned that 20 of the 25 patients had histologically confirmed bladder cancer. Overall, microsatellite analysis with the 13 markers detected genetic alterations in 19 of these 20 cancer patients. Of four patients with inflammation that prompted cystoscopy, two showed molecular changes (LOH, alterations, or both) in the urine, and both had bladder lesions containing atypical cells that were suspicious but not diagnostic for cancer (Table 1). None of the five patients without neoplasia (controls) showed any microsatellite changes.

To confirm that the genetically altered alleles were derived from exfoliated cancer cells, we examined the primary tumors from biopsies of 15 of the 20 cancer patients (in five cases, there was insufficient biopsy material for this analysis). In all patients, the same microsatellite alterations and LOH patterns detected in the urine were also detected in the primary tumor (Table 2). However, in two patients (patients 4 and 8), the urine samples showed LOH or microsatellite alterations that were not present in the biopsies. In both cases, LOH in at least one locus (and loss of the identical allele) was shared between the urine sediment and the primary tumor (Table 2). Conceivably, the urine sample may have contained a more advanced tumor cell clone that was derived from the same progenitor cell but was not sampled by the small biopsy of the tumor.

Cells from the same urine samples were then examined by light microscopy (Fig. 2). Cytologic analysis was performed in a blinded fashion, following normal clinical procedures (*12*), on samples from 18 of the 20 patients with bladder cancer and from 3 of the 5 patients with suspicious lesions but without neoplasia. Neoplastic cells were identified by cytology in 9 of the 18 patients for whom molecular analysis was positive and in 1 patient (patient 9) for whom molecular analysis was negative (Tables 1 and 2).

We have demonstrated that microsatellite analysis can be a powerful tool in the detection of primary bladder cancer. The ease of LOH detection in urine sediment is consistent with recent analysis on urine samples by fluorescence in situ hybridization (FISH) (*13*). Moreover, molecular analysis of patients with multiple tumors has demonstrated that these multiple tumors appeared to arise from a single progenitor cell that seeded and populated the bladder mucosa, potentially accounting for the high risk of recurrence in these patients (*14*). Our observations are compatible with the hypothesis that large areas of transformed bladder mucosa can exist in patients with small neoplasms. Other factors may also contribute to the enrichment of tumor cells in urine; for example, more tumor cells than normal cells may survive storage (*15*). In addition, as tumor surfaces are composed of actively growing cell populations that clonally expand through mechanisms such as loss of adhesion, it is possible that these cells are more readily shed into the urine (*16*).

Our markers enabled us to detect 95% of the bladder cancers in this study, but as new markers are identified, the approach can be expanded and improved (*17*). Although we expected to identify only ~50% of cases, the identification of LOH in addition to microsatellite alterations greatly improved our detection strategy. We also identified an adenocarcinoma of the prostatic fossa (patient

Table 2. Microsatellite analysis of urine sediment. T, tumor (in patients 22 and 23, nonneoplastic tissue); U, urine; L, LOH; S, allele shift (alteration); ●, tumor or biopsy not available. Cytology symbols: N, normal; +, positive for tumor; −, negative for tumor; A, atypia; ND, not done. The chromosomal location of each marker follows the letter D (for example, D16S310, chromosome 16). Additionally, gene loci are ACTBP2 on chromosome 6, FGA on 4, MBP on 18, MJD on 14, and IFNA on 9. Of 25 total patients with clinical suspicion of cancer, 20 had a confirmed diagnosis of cancer by pathology. Of the 20, 19 (95%) had identical clonal alterations in their tumor and urine samples, and at least 9 (45%) were cytologically negative for cancer (2 ND). The five control patients (not shown) had no molecular changes in urine. LOH was observed consistently in urine but was obscured in tumors (for example, patient 17) because only a few neoplastic cells were present in the tumor biopsy or because infiltration with normal lymphocytes occurred.

Patient	Cytology	Molecular changes at marker																			
		ACTBP2		D16S310		FGA		D21S1245		D4S243		D16S476		D9S747		D18S51		MBP		MJD		D9S162		IFNA		D9S171	
		T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U	T	U
1	+						L	L				L		*L	L			L	L				L	L	L	L	L	L
2	ND	●	L								●	L															
3	−							S	S				S														
4	−		L										L					L		L	L	L			L		
5	+	L	L		L	L					S	S			L	L		L	L		L	L				L	L	L	L
6	A												●	S							●	S						
7	+	L	L				L	L					L	L														
8	+		S												L		L	L	L						L	L		
9	+																											
10	A								L			L											L	L			L	L
11	A								●	S					●	L									●	L		
12	ND																		L	L								
13	−								●	L											●	S						
14	N									L																		
15	N																		L									
16	+	L	L				L	L		S	S				L	L							L	L				
17	+		L																		L				L			
18	+	L	L							S	S																	
19	+									●	L																	
20	−						S	S				S	S															
21	ND				●	S																	●	L		●	L		●	L
22	ND																											
23	−																											
24	A						●	L											●	L	●	L						
25	−																											

*LOH was observed in both the urine and the tumor, but an additional alteration was observed only in the tumor (see Fig. 1).

7); perhaps markers commonly deleted in other genitourinary tract neoplasms may facilitate the detection of other neoplasms that exfoliate cells into urine sediment.

Finally, our approach highlights the immediate utility of studies that demonstrate LOH in human cancer and of the development of molecular progression models for clinical detection (*18*). In most of the cases in this study, morphologic and cytologic analyses were not diagnostic. Molecular analysis reliably detected tumors of all grades and stages, including those often missed by cytology. In principle, this molecular approach can be performed at approximately one-third the cost of cytology and does not require exhaustive expert interpretation. Moreover, the entire assay is amenable to nonradioactive, non–gel separation techniques (*19*) and potentially could lead to a reliable, yet inexpensive, molecular screening test.

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9. Frozen tumor tissue was cut into 10-μm sections. All samples (including lymphocytes) were digested with 1% SDS–proteinase K at 60°C for 5 hours, and DNA was extracted by ethanol precipitation. Urine samples were spun at 3000*g* for 5 min and washed twice with phosphate-buffered saline. Each PCR mixture (25 μl) contained 50 ng of DNA template. Primers were obtained from Research Genetics (Huntsville, AL) or synthesized from sequences in the Genome Database (GDB). For microsatellite analysis, one primer was labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]-adenosine triphosphate (New England Nuclear). Microsatellite loci and primer sequences are available from the authors on request. DNA was subjected to 30 to 35 cycles of amplification in a Hybaid (Middlesex, UK) Omnigene TR3 SM2 Thermocycler as follows: 95°C for 30 s, 52° to 60°C for 1 min, and 72°C for 1 min, with a final extension step of 72°C for 5 min. PCR products were separated by electrophoresis in denaturing 8 M urea-polyacrylamide-formamide gels, which were then subjected to autoradiography (*8*).
10. We tabulated the total alterations for each marker tested in all tumors and then grouped, in descending order, the markers most susceptible to alterations that would empirically detect the greatest number of primary tumors. Thus, the 10 most susceptible markers empirically identified at least one alteration in 26 of 50 tumors (52%), 11 identified 56% of tumors, 12 identified 60%, 13 identified 62%, and so forth. Some markers identified alterations only in tumors previously identified by another marker, and 20% of tumors did not demonstrate a single alteration with any marker tested.
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12. Approximately 50 cm^3 of urine was obtained and concentrated by centrifugation on cytospin glass slides or Millipore filters (Burlington, MA). Cells were stained by Papanicolaou stain and visualized under microscopy. Samples were initially screened by a cytotechnologist and reviewed by a cytopathologist. Standard morphologic criteria were used to establish the presence of neoplastic cells.
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Expression of a *Plasmodium* Gene Introduced into Subtelomeric Regions of *Plasmodium berghei* Chromosomes

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Targeted integration of exogenous DNA into the genome of malaria parasites will allow their phenotype to be modulated by means of gene disruption or the stable expression of foreign and mutated genes. Described here is the site-specific integration through reciprocal exchange, and subsequent expression, of a selectable marker gene into the genome of the pathogenic, bloodstage forms of the rodent malaria parasite *Plasmodium berghei*. Stable integration of a single copy of the marker gene (retained for more than 70 generations in the absence of drug pressure) into a nontranscribed subtelomeric repeat array of different chromosomes was observed. Expression of the gene within the subtelomeres indicated that the previously recorded absence of transcription in these regions could be due to a corresponding absence of genes rather than active silencing mechanisms.

Systems enabling the genetic manipulation of malaria parasites will play a crucial role in furthering our understanding of the biology of these protozoa. In turn, this knowledge might contribute to improved rationales for the combat of the disease. Targeted insertion of foreign DNA into the malaria parasite genome will permit the examination of the function of parasite genes and the reintroduction of mutated forms of those same genes. The successful introduction of functional plasmid DNA into malaria parasites has been demonstrated in both transient (*1*) and stable (*2*) procedures, but neither random nor site-directed integration of the introduced DNA into the *Plasmodium* genome has been reported.

We have previously shown that it is possible to stably introduce plasmid DNA containing the gene encoding the bifunctional enzyme dihydrofolate–thymidylate synthase (DHFR-TS) (which confers resistance to the drug pyrimethamine) and additional parasite DNA into bloodstages of *Plasmodium berghei* (*2*). When two different plasmids (pMD223 and pMD221) with the potential to integrate into the genome were introduced in both circular and linear forms, we observed only drug-resistant, transformed parasites that maintained the plasmids episomally. In the experiments described here we introduced linearized pMD223 containing an internal 2.2-kb portion of a 2.3-kb repeat element. This 2.3-kb element is a nontranscribed repetitive DNA sequence specific to the rodent malaria parasite *P. berghei*. It is present in a total copy number of 200 to 300 in the genome, arranged as a head-to-tail array, and located exclusively in the subtelomeric regions of several chromosomes (*3*). Plasmid pMD223 was linearized at a unique Spe I site present in the 2.3-kb repeat. In four separate experiments (T6, T7, T8, and T9), the linear form of pMD223 was electro-

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