Clinical Sensitivity of p53 Mutation Detection in Matched Bladder Tumor, Bladder Wash, and Voided Urine Specimens

James L. Prescott, Ph.D. 1
Jim Montie, M.D. 2
Tom W. Pugh, B.S. 1
Terri McHugh, B.S. 1
Robert W. Veltri, Ph.D. 1

1 Research and Development, UroCor, Inc., Oklahoma City, Oklahoma.
2 Department of Urology, University of Michigan, Ann Arbor, Michigan.

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Address for reprints: James L. Prescott, Ph.D., UroCor Inc., 840 Research Parkway, Oklahoma City, OK 73104; Fax: (405) 290-4083; E-mail: jprescott@urocor.com

James L. Prescott, Ph.D., Tom W. Pugh, B.S., and Robert W. Veltri, Ph.D., are employees of UroCor Inc.

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BACKGROUND. Mutations in the p53 tumor suppressor gene may correlate with an increased risk of recurrence and disease progression in patients with bladder carcinoma. The ability to accurately and sensitively detect p53 mutations in cytology specimens may be of benefit in the treatment of bladder carcinoma patients with superficial, minimally invasive disease.

METHODS. Genomic DNA was isolated from 49 cases, each of which was comprised of matched bladder tumor tissue, bladder wash, and voided urine specimens obtained concurrently at a single institution. The genomic DNA was analyzed for mutations in the p53 tumor suppressor gene using a p53 mutation detection assay. Automated dideoxy sequencing of mutant specimens also was performed.

RESULTS. Of the 49 cases, 29 (59%) showed no evidence of p53 mutations in the tumor, bladder wash, or voided urine specimens. Of the remaining 20 cases, 19 showed evidence of mutations in the tumor. Of these 19 p53 mutant bladder tumors, 16 (84%) were detected in the matched bladder wash and 16 (84%) were detected in the matched voided urine specimens. One case resulted in the detection of mutant p53 in the voided urine and the bladder wash, but not in the tumor. Analysis of the results between tumor tissue and bladder wash or tumor and voided urine showed 84.2% sensitivity, 96.8% specificity, and 91.8% accuracy. Sequence analysis of the mutant cases showed that the mutations detected in the tumor tissue were the same mutations detected in the bladder wash and the voided urine specimens.

CONCLUSIONS. Both voided urine and bladder wash specimens from patients with bladder carcinoma were found to provide a high rate of clinical accuracy for the determination of the p53 gene status in patients with bladder tumors. Cancer 2001; 91:2127–35. © 2001 American Cancer Society.

KEYWORDS: p53 gene, bladder carcinoma, mutation, cytology, tumors.
carcinoma tissue as being an independent prognostic factor for disease progression. There is some degree of correlation between the overexpression of p53 protein and mutations in the p53 gene.

Several reports also have shown that p53 mutations can be detected in the cytology specimens of patients with bladder carcinoma. Sidransky et al. used a cloning and sequencing approach in what to our knowledge is the first report of p53 mutation detection in cytology specimens. Vet et al. were able to detect p53 mutations in cytology specimens using a single-strand conformation polymorphism (SSCP)-based detection method followed by sequencing to confirm the presence of mutations. This report also compared the detection of mutations in tumors with those detected in cytology specimens, and reported a sensitivity of 75% and a specificity of 86%. Vet et al. also showed that the p53 mutations were detected, on average, 8 months prior to disease progression. Recent work by Phillips et al. has further confirmed the detection of p53 mutations in cytology specimens.

The current study was undertaken to determine the clinical sensitivity and specificity of the detection of p53 mutations in bladder cytology specimens compared with concomitantly collected tumor histology specimens from bladder carcinoma patients using a commercially available p53 mutation detection assay (UroCor, Inc., Oklahoma City, OK). In addition, we confirmed that the mutations detected in the tumors and cytology specimens were the same based on dideoxy sequencing of the polymerase chain reaction (PCR) products.

### MATERIALS AND METHODS

#### Patient Samples

The Department of Urology of the University of Michigan provided a total of 49 matched bladder tumor, bladder wash, and voided urine samples collected under an Institutional Review Board-approved protocol with informed consent obtained from each patient. The average age of the patient population was 68.9 years, with a range of 47–86 years. A total of 42 males and 7 females comprised the study group. Cytology specimens were obtained on the same day as the tumor resection, with the exception of samples JM45 and JM50, which were taken 2.5 months and 1 month, respectively, prior to resection. Pathologic and cytologic analyses were performed at the University of Michigan and the results are listed in Table 1. The tumor bank tissue sent to UroCor underwent a second pathology review and the presence of tumor was confirmed in 47 of the 49 specimens by hematoxylin and eosin staining.

| Tumor classification | No. | p53 mutant (% of no.) |
|----------------------|-----|-----------------------|
| Ta                   | 13  | 3 (23%)               |
| CIS                  | 6   | 4 (67%)               |
| T1                   | 9   | 4 (44%)               |
| T2                   | 17  | 6 (35%)               |
| T3                   | 1   | 1 (100%)              |
| T4                   | 1   | 0 (0%)                |
| Uncertain            | 2   | 1 (50%)               |

| Tumor grade | No. | p53 mutant (% of no.) |
|-------------|-----|-----------------------|
| Low grade (1/3) | 14  | 0 (0%)                |
| High grade (2/3 or 3/3) | 35  | 19 (54%)             |

| Bladder wash cytology | No. | p53 mutant (% of no.) |
|-----------------------|-----|-----------------------|
| Negative              | 7   | 0 (0%)                |
| Atypia                | 12  | 1 (8%)                |
| Suspicious            | 11  | 3 (27%)               |
| TCC                   | 19  | 12 (63%)              |

| Voided urine cytology | No. | p53 mutant (% of no.) |
|-----------------------|-----|-----------------------|
| Negative              | 11  | 0 (0%)                |
| Atypia                | 9   | 2 (22%)               |
| Suspicious            | 12  | 5 (42%)               |
| TCC                   | 17  | 9 (53%)               |

TCC: transitional cell carcinoma.

### Genomic DNA Isolation

TRIzol Reagent (Life Technologies, Inc., Grand Island, NY) was used to isolate genomic DNA from frozen, OCT (Miles, Elkhart, IN) embedded tissue sections and frozen, pelleted cells according to the manufacturer’s directions. The isolated genomic DNA was resuspended routinely in 65 μL of deionized water prior to storage at −20°C.

### Polymerase Chain Reaction

PCR amplification of the p53 gene sequences utilized a two-step nested strategy in which the primary PCR amplified a 3296-nucleotide sequence extending from the third intron to the ninth exon of the p53 gene.

This primary amplification product extends from nucleotide position 11416 to 14712 of the human p53 gene as indicated in GeneBank accession number X54156. The thermostable DNA polymerase activity used in all the PCR analyses was Expand™ Hi-Fi (Roche, Molecular Systems Inc., Alameda, CA). The reagent concentrations used in the amplifications were those recommended by the vendor. Approximately 5 μL of the genomic DNA isolated as described earlier were used as templates for PCR reactions with...
The hybridized, double-stranded RNAs then were incubated at 95 °C for 3 minutes and allowed to cool to room temperature. The resulting solutions were incubated at 37 °C for 1 hour. Afterward, hybridization buffer (proprietary to Ambion Inc.) containing formamide was added to each transcription in an equal volume.

The primary PCRs products were diluted 1:1 by the addition of deionized water. One microliter of diluted primary PCR then was used as template for amplification in each of the three secondary PCRs. The three secondary PCRs amplify regions encompassing exon 4, exons 5 and 6, and exons 7 and 8 of the p53 gene, respectively. The exon 4 PCR product extended from nucleotide 12149 to 12452, the exon 5–6 PCR product extended from nucleotide 12944 to 13484, and the exon 7–8 PCR product extended from nucleotide 13858 to 14602 of GeneBank accession number X54156. The oligonucleotide primers used in the secondary PCR reactions included the T7 RNA polymerase promoter sequences at their 5’ ends. The exon 4-containing and exons 7–8-containing PCR products were amplified using the same cycling parameters. These were 2 minutes at 92 °C, followed by 30 cycles of 92 °C for 35 seconds, 57 °C for 35 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes followed by storage at 4 °C for up to 24 hours. The cycling parameters for the exon 5 and 6 PCR product were 2 minutes at 92 °C, 30 cycles of 92 °C for 35 seconds, 63 °C for 35 seconds, and 72 °C for 45 seconds, with a final extension at 72 °C for 10 minutes followed by storage at 4 °C for up to 24 hours.

All secondary PCR reactions were electrophoresed through 1.5% agarose gels containing 0.5 µg/mL ethidium bromide with 1X TBE buffer. Electrophoresis was performed at 8 volts/cm. PCR products were visualized on the Alpha Innotech 2000 image analysis system (Alpha Innotech, Inc. San Leandro, CA). Only secondary PCR products that resulted in robust amplification were continued forward in the analysis.

Mutation Detection

In the nonisotopic RNase cleavage assay (NIRCA) (Ambion, Austin, TX), RNA is transcribed in vitro from the secondary PCR products using T7 RNA polymerase. Approximately 200 ng of the secondary PCR reactions were used as template in the in vitro transcription reactions that also included 40 units of T7 polymerase and 500 µM of each rNTP in a buffer provided by Ambion. The transcription was performed at 37 °C for 1 hour. Afterward, hybridization buffer (proprietary to Ambion Inc.) containing formamide was added to each transcription in an equal volume. The resulting solutions were incubated at 95 °C for 3 minutes and allowed to cool to room temperature. The hybridized, double-stranded RNAs then were cleaved by digestion with RNase T1 and Escherichia coli RNase 1 for 45 minutes at 37 °C. The cleaved digestion products then were displayed by electrophoresis through a 3% NuSieve agarose gel (3:1) (Bio-Whittaker Molecular Applications, Rockland, ME) with 1 X TBE buffer. These products also were visualized using the Alpha Innotech digitized image analyzer. Observation of cleavage products indicated a mismatched base pairing in the heteroduplex RNA, and thus a mutation in the genomic DNA of some of the cells in the sample.

Sequencing

The PCR products containing a mutation were amplified using the protocol described earlier and purified using QIAamp Kit (QIAGEN, Inc., Valencia, CA) following the manufacturer’s directions. Purified PCR products were subjected to automated dideoxy sequencing at the Oklahoma Medical Research Foundation (OMRF) (Oklahoma City, OK) sequencing facility. These were analyzed using MegAlign v3.15 (DNASTAR, Madison, WI) and the histogram traces furnished by OMRF. The location of the mutations was narrowed using the results of the NIRCA. Only sequence differences that were reproducible in both the sense and antisense orientations were scored as mutations.

RESULTS

A total of 49 matched bladder tumor, bladder wash, and voided urines specimens were analyzed in this study. In 47 of the 49 specimens (96%), all 3 samples were collected on the same day. Of the remaining 2 specimens, in 1 case the cytology samples were collected 2.5 months prior to tumor resection and in the second case the cytology samples were collected 1 month prior to tumor resection. The patient demographics were within the expected ranges for bladder carcinoma, with the exception of the male-to-female ratio. The ratio of male to female patients seen clinically was 3:1, whereas in the current study population it was 6:1. This difference was not expected to bias the results of the study.

All specimens were subjected to p53 mutational analysis following UroCor’s clinical standard operating procedure. An in-depth discussion of the procedure can be found elsewhere. Briefly, genomic DNA was isolated from OCT-embedded frozen tissue sections and cytology sediments. The genomic DNA was subjected to a two-step, nested PCR strategy to amplify the 3’ portion of exon 4 into intron 4 (avoiding the high frequency polymorphism at codon 72), exons 5 and 6 including surrounding intron-exon boundaries, and exons 7 and 8 including surrounding intron-exon boundaries. The primers used for the exon-spe-
specific PCR included the T7 RNA polymerase promoter at the 5’ end. The PCR products were used as a template for RNA transcription using T7 RNA polymerase. Because the T7 promoter was at both ends of the PCR products, both sense and antisense RNA is produced. After transcription, the RNA is heat denatured and allowed to cool to room temperature. Because both sense and antisense strands are present in the solution, double-stranded RNA is formed. The double-stranded RNA then is subjected to digestion with RNase 1 and RNase T1 and the products separated by gel electrophoresis. Double-stranded RNA is more refractory to digestion by RNases compared with single-stranded RNA. Any regions of single-stranded RNA, such as those created by a base pair mismatch, are preferentially cleaved to create shorter length RNA molecules that can be resolved by gel electrophoresis.

The tabulated results of the p53 test analysis are shown in Table 2. Of the 49 matched specimens analyzed, 29 (59%) showed no evidence of a p53 mutation in the tissue, bladder wash, or voided urine. Of the remaining 20 matched specimens, 19 (95%) showed evidence of a mutation in the bladder tumor. Of these 19 p53 mutant bladder tumors, 16 mutations (84%) were detected in the bladder wash and 16 mutations (84%) were detected in the voided urine. As can be noted in Table 2, the mutations detected in the bladder wash and voided urine did not exactly overlap, with examples of mutations being detected in the bladder wash but not the voided urine (JM28) and vice versa (JM21). There were two cases of a p53 mutant tumor that was not detected in either cytology specimen (JM25 and JM54). There was one example (JM45) in which the cytology specimens detected a p53 mutation that was not present in the tumor.

The p53 test results presented in Table 2 and the performance characteristics of the test are illustrated in Table 3. Both the tumor versus bladder wash and the tumor versus voided urine analyses resulted in a sensitivity of 84.2% and a specificity of 96.8%. The accuracy of both comparisons was 91.8%, indicating a very high percentage of correct classifications in the cytology specimens compared with the matching tumors.
The distribution of p53 mutations based on the pathologist’s analysis of the specimens generally followed the expected trend of increasing p53 mutations with increasing tumor stage and grade as well as with increasing cytopathology diagnosis (Table 1). Pathologic T2 tumors showed a mutation rate of 35%, which is below the mutation detection rate we observed in the carcinoma in situ and T1 tumors. However, with the low numbers of any individual histologic tumor stage, biasing of the population could be expected to occur. Pathologically low-grade tumors showed no p53 mutations, whereas approximately 50% of the high-grade tumors showed evidence of mutations. The bladder washes also tended to show an increase in the frequency of p53 mutations, with 12 of the 16 bladder washes scored as p53 mutant and being diagnosed cytopathologically as TCC. A similar trend was observed in the voided urine specimens, although only 9 of the 16 mutant specimens were diagnosed as TCC by cytopathology.

To confirm that the mutations observed in the tumor and matched cytology specimens were the same, all mutant specimens were sequenced to determine the exact genetic change. With the exception of JM13 and JM45, the sequencing results match the p53 mutation detection results and the mutations found in the cytology specimens are the same as noted in the tumor (Table 4). In the case of the JM13 tumor sample, two mutations were revealed by sequencing and both were missense mutations. The bladder wash specimen in this case also showed these same mutations. However, the voided urine only had the missense mutations of Arg283->Cys. Because the p53 mutation detection assay is a qualitative assay and is only scored as a mutation detected or no mutation detected, this is a reasonable result. For the JM45 case, no mutation was detected by either the p53 mutation detection assay or by sequencing; therefore, the mutation detected in the cytology samples could not be compared with the tumor. However, the mutation detected in the bladder wash was the same as the mutation in the voided urine specimen.

Analysis of the sequencing histograms was performed to examine the source of the discrepancy for those cases in which the tumor and cytology data did not match. In Figure 1A the sequencing traces from case JM38 are shown with the location of the mutation indicated by an arrow. This figure is used to illustrate the situation in which all three sample types correlate in the mutation detection results, both by p53 mutation detection and by sequencing (Fig. 1A). In this case, the wild-type base at nucleotide 14043 is an adenosine. Both the tumor and the bladder wash have

| Specimen ID | Result | Sequence | p53 analysis | Sequence | p53 analysis |
|-------------|--------|----------|--------------|----------|--------------|
| JM3         | nt 14118 Splice | Same | Mut | Same | Mut |
| JM9         | Arg283->Cys | Same | Mut | Same | Mut |
| JM12        | Gln285->Lys | Same | Mut | Same | Mut |
| JM13        | Asn239->Ser | Same | Mut | Same | Mut |
|             | Arg283->Cys | Same | Same | Same | Same |
| JM15        | Arg196->Cys | Same | Mut | NMD | Mut |
| JM16        | Gln285->Lys | Same | Mut | Same | Mut |
| JM17        | Thr211->Asn | Same | Mut | Same | Mut |
| JM19        | Asp239->Ser | Same | Mut | Same | Mut |
| JM20        | Arg213->Gln | Same | Mut | NMD | Mut |
| JM21        | Gln285->Stop | Same | Mut | Same | Mut |
| JM25        | Arg280->Thr | Same | NMD | NMD | NMD |
| JM26        | Gln285->Stop | Same | Mut | Same | Mut |
| JM27        | Arg156->Pro | Same | Mut | Same | Mut |
| JM28        | Ser96->Cys | Same | Mut | Same | Mut |
| JM29        | Arg280->Lys | Same | Mut | Same | Mut |
| JM38        | nt 13964 | Same | Mut | Same | Mut |
|             | Asn239->Ser | Same | Same | Same | Same |
| JM45        | NMD | Gln285->Lys | Mut | Gln285->Lys | Mut |
| JM46        | Arg238->Trp | Same | Mut | Same | Mut |
| JM54        | Arg238->Gln | NMD | NMD | NMD | NMD |

Mut: mutation detected; NMD: no mutation detected.
a relatively high percentage of mutant alleles, in which the adenosine is mutated to a guanosine. Because it samples only the exfoliated cells of the urothelial surface, the voided urine specimen has a lower percentage of cells with mutant alleles but still has a sufficiently large enough number of mutant alleles to be detected by either methodology. As an example of a discordant case, JM54 is used because both the bladder wash and voided urine specimens did not show the mutation found in the tumor (Fig. 1B). In the JM54
tumor, nucleotide 14070 was found to have mutated from adenosine to guanosine (Fig. 1B, arrow, top panel). Examination of the same nucleotide in the bladder wash (middle panel) and voided urine (bottom panel) show that these samples lacked the presence of the mutated allele. We concluded from these data that the discordant results were not due to a failure of the *p53* mutation detection assay, but rather were the result of a sampling problem most likely due to the histologic architecture of the tumor and the availability of the tumor cells to be exfoliated into the bladder lumen.

**DISCUSSION**

Identification of those patients at highest risk for the recurrence and progression of superficial TCC should allow for the selection of an earlier and more appropriate treatment regimen. Independent studies have shown a correlation between overexpression and mutation of the *p53* tumor suppressor gene and poor prognosis in patients with bladder carcinoma.\(^9\)\(^{-13}\) However, there are other studies that do not support a relation between *p53* overexpression and bladder carcinoma.\(^14\)\(^{,15}\) This lack of consistency in the results is likely attributable to several factors, not the least of which is the lack of a standardized immunohistochemistry protocol that has been validated in several laboratories.\(^16\)\(^{,17}\)

Bladder wash and voided urine specimens have been used routinely for several years to detect bladder carcinoma via Papanicolaou cytopathologic visual examination.\(^18\) It therefore is rational to develop new tests that are able to detect the genetic changes that accompany bladder carcinoma in these same exfoliated cells. To our knowledge, there are numerous reports of the molecular detection of *p53* mutations in urine cytology specimens\(^5\)\(^{-7}\) but these methods are more cumbersome and not compatible with the technical performance requirements of routine, high throughput, reference laboratory procedures.

There are at least two important determinates of the clinical performance of a molecular test: first, the technical performance of the test in terms of reproducible detection of all relevant mutations, and second, the clinical specimen type in which the test can be performed reliably. Previously, this *p53* mutation detection assay was shown to detect all possible point mutations including the +1 and −1 frameshift mutations.\(^9\) Using cloned and defined point mutations and known wild-type human genomic DNA, it also was determined that this assay will detect a mutation when it is present in 3–5% of the alleles being assayed (data not shown). This assay also surveys an area of the *p53* gene responsible for > 95% of the reported *p53* mutations.\(^9\)\(^{,19}\)

The second important determinant of a successful molecular test its compatibility with clinical specimen type (in this case, cytology specimens). All clinical specimens, regardless of source, contain normal cells and therefore the test must have a high enough sensitivity to detect genetic changes in this mixture. In the case of bladder cytology, the specimen is sampling a large portion of the entire urothelial surface of the bladder. This can be both a benefit and a drawback. The benefit is that the specimen is not restricted to a specific site as is a biopsy and there may be a mutation in another tumor foci; however, the drawback is that the majority of the urothelial surface can be expected to have genetically and phenotypically normal cells and thus may overwhelm any signal generated from the mutant cells. The current study was designed to address this problem by using synchronously collected histologic tumors and cytology specimens to determine the correlation between those mutations found in the histologic tumor versus the autologous cytology specimen.

The results of the current study demonstrate that molecular analysis of cytology specimens provides a very accurate (91.8%) method with which to detect *p53* mutations without the need for a histologic tumor tissue specimen. Both bladder wash and voided urine specimens provide a viable source of clinical material for the molecular detection of *p53* mutations. The hypothesis prior to the current study was that the more invasive specimen type, the bladder wash specimens, would show a slightly higher sensitivity than voided urine specimens due to bladder washes being directed more toward the surface of tumor foci. However, both specimen types proved to be equally suited and gave the same results, although in nonoverlapping sets. A previous report by Vet et al.\(^6\) showed a relatively similar sensitivity (75%) and specificity (86%). These differences may be related to sample size (49 specimens vs. 26 specimens) as well as the technology used to detect the mutations (NIRCA vs. SSCP).

There are several potential different outcomes from this type of matched analysis and we believe the results of the current study have demonstrated the majority of them. Two, specimens in particular, Cases JM25 and JM54, both were *p53* mutant tumors in which the cytology specimens showed no evidence of mutation by either the *p53* mutation detection assay or dideoxy sequencing. A similar result was observed by Vet et al.\(^6\) in a study in which they demonstrated that the *p53* mutant cells were not exposed to the lumen of the bladder, thus preventing the release of the cells for analysis. These cases represent one of the limitations of using cytology specimens for genetic analysis because the genetically defective cells may
not be exfoliated or accessible by the sampling procedure employed. Another case, case JM45, presented the opposite situation in that both the bladder wash and the voided urine specimens were positive for the identical \( p53 \) mutation yet the tumor showed no evidence of a mutation. This patient presented with multifocal carcinoma in situ and the cytology specimens were collected several months prior to resection. It is possible that the \( p53 \) mutant tumor was not the tumor that was used for analysis. We believe this case illustrates another benefit of using cytology specimens, in that the specimen analyzed is a sampling of the majority of the urothelial surface, whereas a section from a biopsy or tumor resection may miss the critical surrounding cells. In addition, Cases JM21 and JM28 represent the final discordance possibility, in which the tumor and one cytology specimens is mutant and the other cytology specimen has no mutation detected. In both these cases, as for all cases studied, the sequencing data agree with the \( p53 \) mutation detection assay results. The cytology report for Case JM21 for both the bladder wash and voided urine specimens was a cytopathologic diagnosis of TCC, indicating that tumor cells were observed in the specimens. To our knowledge, there remains no satisfactory explanation for this discrepancy. In Case JM28, mentioned earlier, the voided urine cytology report was negative for malignancy, explaining the results for both molecular technologies.

Closer examination of the distribution of the \( p53 \) mutations stratified by pathology indicates that all the mutant tumors were of high grade (\( \geq \) Grade 2/3). This is in agreement with published reports regarding the correlation between \( p53 \) mutations and higher grade tumors.\(^{20,21}\) Analysis of the \( p53 \) mutations by cytopathologic diagnosis also shows that no \( p53 \) mutations were detected in the negative cytology specimens. This result supports the hypothesis that the most clinically relevant utility of this test will be as a reflex from a positive cytopathology finding because the lack of malignant cells in a specimen will likely present a negative \( p53 \) test result. These data also illustrate that the utility of \( p53 \) mutation detection is not suitable for the detection or diagnosis of bladder carcinoma because only 19 of the 49 histologically confirmed tumors examined in the current study would have been detected.

Finally, the results of the current study showed that molecular detection of \( p53 \) mutations in bladder cytology specimens is a sensitive method with a high positive predictive value with which to determine the \( p53 \) gene status of bladder tumors. This assay can be applied to nonformalin-preserved bladder washes and noninvasive voided urine samples with equal accuracy provided the patient has an active case of bladder carcinoma as evidenced by a positive cytopathology finding. Because the current study is technical in nature, based on the concomitant analysis of fresh-frozen material, there was no opportunity to perform matched immunocytochemical, immunohistochemical, and molecular assays. We believe that appropriately designed, multicenter, retrospective, and prospective clinical trials must be performed using rigorously standardized clinical and laboratory protocols. Such Institutional Review Board-approved studies currently are underway using cytologic specimens to determine the clinical utility of the molecular detection of \( p53 \) mutations in patients with active, superficial bladder carcinoma.

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