Development of a Multiplexed Urine Assay for Prostate Cancer Diagnosis

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BACKGROUND: Several studies have demonstrated the value of DNA methylation in urine-based assays for prostate cancer diagnosis. However, a multicenter validation with a clinical prototype has not been published.

METHODS: We developed a multiplexed, quantitative methylation-specific polymerase chain reaction (MSP) assay consisting of 3 methylation markers, GSTP1, RARB, and APC, and an endogenous control, ACTB, in a closed-tube, homogeneous assay format. We tested this format with urine samples collected after digital rectal examination from 234 patients with prostate-specific antigen (PSA) concentrations ≥2.5 μg/L in 2 independent patient cohorts from 9 clinical sites.

RESULTS: In the first cohort of 121 patients, we demonstrated 55% sensitivity and 80% specificity, with area under the curve (AUC) 0.69. In the second independent cohort of 113 patients, we found a comparable sensitivity of 53% and specificity of 76% (AUC 0.65). In the first cohort, as well as in a combined cohort, the MSP assay in conjunction with total PSA, digital rectal examination status, and age improved the AUC without MSP, although the difference was not statistically significant. Importantly, the GSTP1 cycle threshold value demonstrated a good correlation (R = 0.84) with the number of cores found to contain prostate cancer or premalignant lesions on biopsy. Moreover, samples that exhibited methylation for either GSTP1 or RARB typically contained higher tumor volumes at prostatectomy than those samples that did not exhibit methylation.

CONCLUSIONS: These data confirm and extend previously reported studies and demonstrate the performance of a clinical prototype assay that should aid urologists in identifying men who should undergo biopsy.

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In 2007, prostate cancer will be diagnosed in an estimated 218,890 men in the United States, which is the highest incidence among all noncutaneous malignancies. Prostate cancer is the third leading cause of cancer mortality among men. Men who present with increased prostate-specific antigen (PSA) concentrations or abnormal digital rectal examination (DRE) findings may be at risk for prostate cancer and are candidates for prostate biopsy. The low specificity of PSA is well documented, however. Therefore, prostate cancer screening would benefit from a confirmatory test with a higher specificity that could be used in conjunction with PSA testing to determine which patients should undergo biopsy.

Several studies have identified DNA methylation markers in the urine of men with prostate cancer as the basis for a confirmatory test. Cairns et al. (2) reported that, when patients had methylation of GSTP1 detected in their tumor, 27% of cases were positive for DNA methylation in voided urine specimens. Jeronimo et al. (3) later showed sensitivity in urine of 23%. Gonzalgo et al. (4) demonstrated that GSTP1 methylation could be detected in the postbiopsy urine specimens of 39% of patients with prostate cancer.

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Rogers et al. (5) later demonstrated a high concordance between postbiopsy and post-DRE urinary samples for methylation of GSTP1, APC, and EDNRB. Goessl et al. (6) demonstrated a sensitivity of 73% at a specificity of 98% in urine sediments collected after prostate massage. Additional markers could potentially increase the sensitivity. For example, more recently, Hoque et al. (7) found that a combination of CDKN2A (formerly p16), PSCD2 (formerly ARF), MGMT, and GSTP1 could theoretically enable the detection of 87% of prostate cancers at 100% specificity. In that study, GSTP1 alone demonstrated a sensitivity of 48% at a specificity of 100%. A study by Roupret et al. (8) examined GSTP1, RARB, APC, and RASSF1. The authors found a sensitivity of 86% at a specificity of 89%; however, they used a 1-min prostate massage and bladder catheterization, which would hinder widespread adoption.

In this report, we sought to determine the performance of a prototype assay in a multicenter validation study using typical prostate manipulation and sample acquisition scenarios. We also examined correlations between methylation and various clinical parameters.

Materials and Methods

Patients and Urine Collection

Urine samples were obtained from 6 American and 3 European sites: Arkansas Urology, Little Rock, AR; Mayo Clinic, working through Mayo Validation Support Services, Rochester, MN; Pacific Rim Pathology Medical Corporation, San Diego, CA; Arizona Cancer Center, Tucson, AZ; Eastside Urology, Snellville, GA; Advanced Clinical Therapeutics, LLC, Tucson, AZ; Innsbruck Medical University, Department of Urology, Innsbruck, Austria; Graz Medical University, Department of Urology, Graz, Austria; and PharmaTrials, Inc., Warsaw, Poland. The respective institutional review boards approved study protocols, and all study subjects provided written informed consent. The specimens were collected prospectively from August 2006 to March 2007. The 2 cohorts of accrued specimens were unique with respect to patients, although they came from overlapping institutions (none of the patients was included in both cohorts).

To determine stability of methylated DNA in urine under different storage (shipment) conditions, 2 accrual protocols were used for urine collection as depicted in Fig. 1. According to the first protocol, 142 whole urine samples stored for up to 5 days at 4 °C between urine collection and sedimentation procedures were obtained from 9 sites. Specimens were delivered to Veridex on 4 °C cold packs as “neat” urines and were immediately sedimented on site. According to the second protocol, 113 urine specimens were collected and sedimented by sites within 4 h from collection time and delivered to Veridex as frozen sediments at −20 °C. In a final multicenter study, 21 urine samples were excluded from the first cohort for the absence of information on recent PSA concentrations.

The study population for this prototype quantitative methylation-specific polymerase chain reaction (MSP) assay consisted of 255 men with no previous history of prostate cancer and a single follow-up biopsy. In 234 of 255 specimens, the average serum PSA concentration was 5.9 (SD 3.0) μg/L (median 5.44, range 2.5–27.9); average age was 64 (9) (median 66, range 45–90). For the subject group studied, 241 of 255 specimens yielded sufficient DNA for analysis, corresponding to an informative specimen rate of 94.5%. For 241 subjects, 113 biopsies were positive for prostate cancer and 128 were negative. All prostate cancer cases found were Gleason grades 4–6 (67%) and 7–9 (33%).

Sample Preparation

Urine samples (20–120 mL) were collected after digital rectal examination (DRE). All samples were processed using the same sample preparation protocol. After 10-min centrifugation at 3000g, urine sediments were washed with cold PBS and then subjected to DNA extraction with the Puregene Kit (Qiagen). After DNA extraction, DNA was quantified using the Picogreen DNA Quantification Assay (Molecular Probes/Invitrogen). DNA was modified using the EZ-DNA Methylaton Kit (Zymo Research Corp.) and eluted in 50 μL volume. We used 5 μL modified DNA per reaction for real-time quantitative methylation-specific PCR analysis on the SmartCycler® platform (Cepheid).

MSP

We selected 3 methylation markers for a multiplexed PCR assay in a nested (2-round) format, namely, GSTP1, RARB, and APC. For determining failure rate as a cause of insufficient DNA amount, we used ACTB as an internal control. In each run, universally methylated DNA (Chemicon International) was integrated as an internal control. In each run, universally methylated DNA (Chemicon) was used as a positive control as well as standard curve material. Universally unmethylated DNA (Chemicon) was used as a negative control. Primer and probe oligonucleotide sequences for GSTP1, RARB, APC, and ACTB used in a nested multiplexed MSP are listed in Supplemental Tables 1 and 2 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue5.

Nested PCR Protocol

Briefly, 5 μL first-round PCR master mix and 5 μL bisulfite-modified DNA were added to each SmartCap tube. After a quick spin, tubes were placed in the Smart
Cycler instrument, and PCR was run as follows: denaturation at 94 °C for 2 min, followed by 18 cycles of denaturation at 92 °C for 20 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s, and final extension at 70 °C for 5 min.

The first-round PCR master mix contained 4 sets of outer primers at 0.05 μmol/L each for GSTP1, RARB, and APC and 0.04 μmol/L for ACTB; 1 μL of 10× Magic buffer (16.6 mmol/L ammonium sulfate, 67 mmol/L Trizma, pH 8.8, 6.7 mmol/L MgCl2, and 10 mmol/L mercaptoethanol), 0.5 U Taq (Ab) polymerase, and 0.1 mmol/L dNTP mix.

For the second-round PCR setup, 15 μL second-round PCR master mix was added to the SmartCap reservoir to a final volume of 25 μL. The lid was snapped into place and, after a quick spin, inner PCR reactions were run on the Cepheid platform for an additional 40 cycles under the following cycling conditions: denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 20 s and annealing at 59 °C for 30 s with data collection, and final extension at 70 °C for 5 min.

The second-round PCR master mix contained 4 sets of inner Scorpion probes/primers at 0.4 μmol/L each for GSTP1, RARB, and APC and 0.24 μmol/L for ACTB; 1.5 μL of 10× Magic buffer, 1.5 U Taq (Ab) polymerase, and 1 mmol/L dNTP mix.

**DATA ANALYSIS**

For methylation analysis, we used cycle threshold (Ct) values from 3 markers and ACTB. A cutoff value for each marker was determined. If one Ct value from the set of 3 methylation markers was below the defined cutoff, the sample was considered methylated. Samples with Ct values above the defined cutoffs were scored as unmethylated. No test rate was calculated based on the Ct cutoff for ACTB (internal control).

AUC values were calculated based on ROC analysis. For single-marker analysis, we generated AUC values using MedCalc software, and for different combinations of multiple markers, we applied logistic regression model in S-Plus statistical software.

**Results**

**PERFORMANCE IN 2 INDEPENDENT PATIENT COHORTS**

We sought to determine the performance of our methylation assay in 2 different cohorts to assess stability of methylated DNA in urine specimens. The first cohort comprised 121 urine samples that were collected after
prostate massage and that were shipped at 4 °C to Veri-
dex within 5 days of collection (Fig. 1). The samples
were sedimented and processed at Veridex. The second
cohort comprised 113 urine samples that were col-
clected after prostate massage and sedimented within
4 h at the clinical site according to protocols from peer-
reviewed articles (2, 3, 7). The sediments were then
shipped to Veridex. Table 1 shows a comparison of
clinicopathologic parameters of the 2 patient cohorts.
No statistically significant differences were found be-
tween the 2 cohorts by use of Student t test, with the
exception of race/ethnicity (P = 0.01). Moreover, be-
cause the sample acquisition protocols for the 2 cohorts
were drastically different, these cohorts were not in-
tended to be a training and validation set, but rather
were used to assess urine stability.

Analysis of the first cohort comprising 54 patients
with cancer and 67 patients with biopsies negative for
cancer is shown in Fig. 1 (left pathway). GSTP1, RARB,
and APC each showed modest sensitivity but high spec-
ificity (Table 2). The 3-marker combination showed a
sensitivity of 55% and a specificity of 80%, with an
AUC value of 0.69. The no-test rate (the percentage of
samples for which a test result could not be delivered,
typically due to inadequate yield of DNA) was 8%. The
specificity of the markers was evident when the patient
cohort was stratified according to having 0, 1, 2, or 3
markers positive in the assay (Fig. 2A). The data dem-
onstrate that cancer prevalence increases to 85% and
80% when 2 or 3 markers, respectively, are positive in
the assay, thus suggesting that there is a higher likeli-
hood of having cancer when 2 or more markers are
present in the assay. We note that this prevalence is
high because we enriched for cancer patients during
our collection process to generate more robust subse-
quent data analyses. This type of analysis also under-
scored the high specificity of the individual markers, as
the cancer prevalence for populations where GSTP1,
APC, or RARB was positive was found to be 85%, 66%,
and 73%, respectively (Fig. 2B).

To address urine stability over storage time, we
examined a subset of 73 patient samples from the first
cohort (left pathway in Fig. 1). This subset consisted
only of samples that were shipped from the sites within
3 days of collection and comprised 30 patients positive
for prostate cancer and 43 patients negative by biopsy.
We found that the overall performance, especially
specificity, improved in this subset, exhibiting a sensi-
tivity and a specificity of 50% and 90%, respectively
(AUC 0.76) using 3 markers (Table 2). We conclude
that stability of urine samples within 3 days of storage
at 4 °C is a key factor in achieving optimal performance
of the assay.

To verify both this conclusion and the assay per-
formance in an independent cohort of samples, we an-
alyzed 113 specimens (57 patients positive for prostate

Table 1. Clinical parameters of the 2 patient cohorts (n = 255).

<table>
<thead>
<tr>
<th>Clinicopathologic characteristics</th>
<th>Cohort 1 (neat urine samples), 121 patients</th>
<th>Cohort 2 (sediments), 113 patients</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biopsy status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>54 (45)</td>
<td>57 (50)</td>
<td>0.38</td>
</tr>
<tr>
<td>Negative</td>
<td>67 (55)</td>
<td>56 (50)</td>
<td></td>
</tr>
<tr>
<td>Mean total PSA µg/L</td>
<td>6.0</td>
<td>5.9</td>
<td>0.61</td>
</tr>
<tr>
<td>Mean age</td>
<td>64.4</td>
<td>66.0</td>
<td>0.59</td>
</tr>
<tr>
<td>DRE results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>21 (24)</td>
<td>28 (37)</td>
<td>0.06</td>
</tr>
<tr>
<td>Normal</td>
<td>67 (76)</td>
<td>47 (63)</td>
<td></td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean score</td>
<td>6.3</td>
<td>6.3</td>
<td>0.61</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>105 (97)</td>
<td>100 (88)</td>
<td>0.01</td>
</tr>
<tr>
<td>Other</td>
<td>3 (3)</td>
<td>13 (12)</td>
<td></td>
</tr>
</tbody>
</table>

Data are n (%) unless noted otherwise. For each patient cohort, we were not able to obtain full clinical information on every patient; therefore, the number of patients for which we had information on that particular parameter is shown along with the value. We performed a t test to determine whether there were statistically significant differences between the 2 cohorts for each parameter.
cancer and 56 patients negative by biopsy) from the right pathway in Fig. 1 (sedimented at the clinical sites within 4 h). The assay showed comparable performance in this cohort, with a sensitivity and specificity of 53% and 76%, respectively (AUC 0.65), using 3 markers (Table 2). The no-test rate was determined to be 6%.

A combination of clinical parameters, rather than only PSA, is typically used to determine who should undergo a biopsy. Thus, we compared the performance of our MSP assay with that of PSA plus DRE status plus age (Fig. 3). We were able to obtain information on these 3 clinical parameters from 88 patients in cohort 1 (left pathway in Fig. 1). ROC curves demonstrated that the MSP assay or the combination of clinical parameters had equivalent performance (AUC 0.63 for each), but that the MSP assay in conjunction with the 3 clinical parameters showed an increase in the AUC value to 0.66. However, this increase was not statistically significant ($P = 0.28$). When both cohorts (left and right pathways of Fig. 1) were combined, we were able to obtain information on these 3 clinical parameters from 163 patients. Once again, the MSP assay or the combination of clinical parameters had equivalent performance (AUC 0.60 for each). However, the MSP assay in conjunction with the 3 clinical parameters showed an increase in the AUC value to 0.65, but with no statistical significance observed ($P = 0.12$). We note that the AUC of the MSP assay in this combined cohort was lower than in either of the individual cohorts (Table 2) because the differential sample acquisition methods resulted in differences in the ideal cutoffs for each marker. In a true clinical assay, a standard procedure would be used, and such cohorts would not be combined. We conclude that clinicians can derive similar benefit from the MSP assay as from the combination of age, PSA, and DRE status.

Many of the false positives (in both patient cohorts) in our assay had an abnormal DRE and/or multiple markers that were positive. Thus, a potential explanation for the false positives is sampling error at the time of prostate biopsy. For 3 of the sites, we were able to obtain information regarding the number of cores taken for each patient’s biopsy. The average numbers (SD) of cores taken were 9.1 (2.3), 11.3 (3.8), and 12.4 (4.9) for Poland, Arizona, and Minnesota, respectively, and the distributions are shown in Supplemental Fig. 1 in the online Data Supplement. We found that the specificity of the markers was higher when more cores were examined in the biopsy. We conclude that an op-

### Table 2. Sensitivity and specificity of **GSTP1**, **RARB**, **APC**, and combinations.

<table>
<thead>
<tr>
<th>Gene or combination</th>
<th>Sensitivity, % (95% CI)</th>
<th>Specificity, % (95% CI)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cohort 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GSTP1</strong></td>
<td>33 (20–48)</td>
<td>95 (90–100)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>RARB</strong></td>
<td>40 (26–56)</td>
<td>84 (75–93)</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>APC</strong></td>
<td>36 (22–50)</td>
<td>91 (82–98)</td>
<td>0.59</td>
</tr>
<tr>
<td><strong>GSTP1 and APC</strong></td>
<td>51 (37–65)</td>
<td>89 (81–97)</td>
<td>0.68</td>
</tr>
<tr>
<td><strong>All 3</strong></td>
<td>55 (41–70)</td>
<td>80 (70–90)</td>
<td>0.69</td>
</tr>
<tr>
<td><strong>Cohort 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GSTP1</strong></td>
<td>36 (24–49)</td>
<td>91 (83–98)</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>RARB</strong></td>
<td>29 (17–419)</td>
<td>91 (83–98)</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>APC</strong></td>
<td>51 (38–64)</td>
<td>83 (73–93)</td>
<td>0.62</td>
</tr>
<tr>
<td><strong>GSTP1 and APC</strong></td>
<td>53 (40–66)</td>
<td>80 (69–90)</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>All 3</strong></td>
<td>53 (40–66)</td>
<td>76 (65–87)</td>
<td>0.65</td>
</tr>
<tr>
<td><strong>Samples shipped within 3 days</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GSTP1</strong></td>
<td>27 (10–44)</td>
<td>100</td>
<td>0.66</td>
</tr>
<tr>
<td><strong>RARB</strong></td>
<td>31 (13–49)</td>
<td>93 (85–101)</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>APC</strong></td>
<td>35 (16–53)</td>
<td>98 (93–102)</td>
<td>0.64</td>
</tr>
<tr>
<td><strong>GSTP1 and APC</strong></td>
<td>50 (31–69)</td>
<td>98 (93–102)</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>All 3</strong></td>
<td>50 (31–69)</td>
<td>90 (81–99)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Cohort 1: 121 urine samples shipped to Veridex within 5 days of collection; 54 biopsy positive and 67 biopsy negative. Cohort 2: 113 urine sediments (sedimented within 24 h prior to shipping to Veridex); 57 biopsy positive and 56 biopsy negative. Shipped within 3 days: 73 urine samples shipped to Veridex within 3 days of collection; 30 biopsy positive and 43 biopsy negative.
CORRELATIONS WITH CLINICOPATHOLOGIC PARAMETERS

The clinical utility of the assay would be enhanced if it correlated with diagnostic or prognostic features. For a subset of the cancer samples, we were able to obtain extensive biopsy and prostatectomy information. We first investigated possible correlations with the number of cores found to contain malignant or premalignant lesions on biopsy. The importance of this parameter has been demonstrated by several investigators (12–15). We found that the Ct value of GSTP1, a measure of the relative methylation level, showed a good Pearson correlation (R = 0.84, P = 0.008) with the number of positive cores on biopsy (Fig. 4). Importantly, neither the PSA concentration (as depicted by the size of the bubble in Fig. 4) nor the RARB and APC Ct values correlated with the number of positive cores (data not shown). We next investigated whether cancer patient samples that were methylated for either of the markers differed in their tumor volumes from unmethylated cancer patient samples. We found that samples methylated for GSTP1 or RARB, but not APC, typically had a higher tumor volume than unmethylated samples (P < 0.001) (see Supplemental Fig. 2, A and B, in the online Data Supplement).

We then examined correlations between our methylation assay and patient age. We found that methylation levels for GSTP1 and RARB, but not APC, were higher (lower Ct value) in cancer patients 80 years or older compared with cancer patients who were <60 years old (P = 0.04 and P < 0.001, respectively) (see Supplemental Fig. 3, A and B, in the online Data Supplement).

Discussion

In this report, we demonstrate the first multicenter validation of a prototype methylation assay in urine samples with 2 independent patient cohorts. The sensitivity and specificity in the first cohort (n = 121) were 55% and 80%, respectively, whereas the sensitivity and specificity in the second cohort (n = 113) were 53% and 76%. These data and the markers chosen are in good agreement with previous reports (2–8). We note that the sensitivities of GSTP1 (43%) and APC (52%) in these urine sediments were similar to those seen by Hoque et al. (7), who demonstrated a sensitivity of 48% for both GSTP1 and APC. We also note that the sensitivities of APC (52%) and RARB (56%) in these urine sediments were remarkably similar to those seen by Roupret et al. (8), who demonstrated sensitivities of 51% and 62% for APC and RARB, respectively. The fact that the 2 cohorts were collected in different ways (Fig. 1) but showed comparable performance suggests that DNA methylation assays may be quite robust in urine samples.

Two additional features underscore the value of the assay. First, it has the potential for higher specificity (Table 2) than assays employing the expression-based
marker PCA3 (16, 17). Van Gils et al. (16) reported PCA3 sensitivity of 65% and specificity of 66% (AUC 0.66) in 534 patients (PSA > 4 μg/L) including 174 men (33%) with cancer-positive biopsy. Marks et al. (17) observed sensitivity of 58% and specificity of 72% (AUC 0.68) in a population study of 233 patients (PSA > 2.5 μg/L) with cancer representation at 27% after repeat biopsy. Second, our prototype MSP assay was found to have a higher sensitivity compared to the assays employing the fusion-based marker TMPRSS2: ERG, which, as reported by Hessels et al. (18), had a sensitivity of 37% in 108 patients including 78 men (72%) with cancer-positive biopsy. The importance of these performance features may be magnified at lower PSA concentrations, where biopsies are not routinely performed but may be considered (9–11, 19). Preliminary data suggest correlation with number of positive cores found upon biopsy (Fig. 4), tumor volume at prostatectomy (see Supplemental Fig. 2 in the online Data Supplement), and age (see Supplemental Fig. 3 in the online Data Supplement), which raises the possibility that our prototype assay may be of value for determining cancer risk as well as facilitating diagnosis.

The fact that the GSTP1 methylation level correlates with both the number of positive cores found upon biopsy and tumor volume is consistent with earlier reports that the percent or number of cores positive for cancer is strongly predictive of tumor volume.
Urine Assay for Prostate Cancer Diagnosis

that, in biopsies, GSTP1 methylation levels correlate with tumor volume. It is possible that markers used in this assay may also have prognostic value, given the increased detection of cancer in biopsy tissue when GSTP1 methylation was used in combination with histology (28). Thus, risk assessment may be improved by using this assay as an adjunct to current screening tools.

The high specificity of these markers may aid in refining current algorithms for risk assessment in prostate cancer (25–27). For example, cancer prevalence in the population of patients having PSA <4 μg/L when both cohorts were combined (n = 70) was 44%. We note that this prevalence is higher than reported by others (9) because we enriched for cancer patients during our collection process to generate subsequent robust data analyses. Importantly, the cancer prevalence in the population of patients having PSA <4 μg/L and positive GSTP1 methylation was 67%. These data are consistent with the increased detection of cancer in biopsy tissue when GSTP1 methylation was used in combination with histology (28). Thus, risk assessment may be improved by using this assay as an adjunct to current screening tools.

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