DNA Integrity Assay: A Plasma-Based Screening Tool for the Detection of Prostate Cancer

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Abstract

Purpose: The aim of this study was to evaluate the utility of the DNA integrity assay (DIA) as a plasma-based screening tool for the detection of prostate cancer.

Experimental Design: Blood samples were collected from patients with biopsy-proven prostate cancer prior to prostatectomy (n = 123) and processed as two-spin plasma preparations. The three control groups included: males <40 years old with no history of cancer (group 1, n = 20); cancer-free postprostatectomy patients (group 2, n = 25), and patients with a negative prostate biopsy (group 3, n = 22). DNA in plasma preparations were isolated, hybrid-captured, and DNA fragments (200 bp, 1.3, 1.8, and 2.4 kb) were multiplexed in real-time PCR. A baseline cutoff was determined for individual fragment lengths to establish a DIA score for each patient sample.

Results: Patients with prostate cancer (86 of 123; 69.9%) were determined to have a positive DIA score of ≥7. The DIA results from control groups 1, 2, and 3 showed specificities of 90%, 92%, and 68.2%, respectively. Of the patients with negative age-adjusted prostate-specific antigen (PSA) and prostate cancer, 19 of 30 (63%) had a positive DIA score. The area under the receiver operating characteristic curve for DIA was 0.788.

Conclusion: While detecting 69.9% of those with prostate cancer, DIA maintained an overall specificity of 68.2% to 92%, a range favorably comparable to that currently accepted for PSA (60-70%). The variability in specificity between control groups is likely explained by the established 19% to 30% detection of prostate cancer on subsequent biopsies associated with control group 3. DIA detected 63% of the prostate cancers undetected by currently accepted PSA ranges.

Cell-free DNA released from tumors into bodily fluids has become a promising biomarker template for the development of new clinical assays (1–4). Such assays have focused on the identification of specific genetic changes associated with particular neoplastic pathways including the detection of microsatellite alterations (5, 6), methylation status of genes (7–9), mutational events (10–12), and other genetic aberrations (13). A number of groups have also shown that quantification of cell-free DNA can act as an indicator of tumor presence (14–17) but might not discriminate between benign and malignant lesions in all cancers (17).

The DNA integrity assay (DIA) was originally characterized in DNA isolated from stool samples and was shown to be indicative of colorectal tumor presence (18). Subsequent experiments have shown that large fragments of DNA were released from tumor cells via tumor necrosis facilitating the maintenance of DNA integrity (19). In contrast, cell death in normal tissues is closely regulated by apoptosis in which the processing of DNA results in fragments between 180 and 210 bp. Hence, DIA is a qualitative rather than a quantitative assay. The potential differential processing of DNA in normal and cancer states has been explored in the plasma of females with cancer, presenting the first indication that DIA may provide a simple measure for the detection of cancer (20).

Prostate cancer is the most commonly diagnosed malignancy in males in which an elevated level of prostate-specific antigen (PSA) or an abnormal digital rectal exam is often the first indication of an underlying disease state (21). The clinical success of PSA as a marker for prostate cancer has been limited by its lack of specificity because the detection of elevated levels of PSA can occur in a number of conditions that affect the prostate gland including benign prostatic hypertrophy and prostatitis (22). In addition, ~25% of men with prostate cancer do not display elevated levels of serum PSA (23). Although the cutoff point to trigger further testing remains controversial, investigators have used various PSA calculations to improve its clinical utility, including age-adjusted PSA, PSA.
velocity, density, and doubling time (24–26). Regardless of the PSA cutoff, an abnormal digital rectal exam is another common trigger for transrectal ultrasound guided biopsy of the prostate. However, transrectal ultrasound biopsy is reported to miss between 19% and 30% of prostate cancers, adding to the number of cancers remaining undetected in screening. It is clear that additional tests are needed to complement those routinely used in clinical practice. This study was designed to evaluate the utility of the DIA as a plasma-based screening tool for the detection of prostate cancer.

**Materials and Methods**

**Patient selection.** Blood samples (15 mL) were obtained from consenting patients as part of an ongoing Institutional Review Board–approved protocol for prostate cancer at the Lahey Clinic. Samples were collected from 123 patients with biopsy-proven prostate cancer prior to undergoing radical prostatectomy at our institution. Three control groups were designed to optimize the DIA information in a male-based population seen at the clinic: control group 1 (n = 20), a low-risk male donor group <40 years of age with no history of cancer and a negative family history of prostate cancer, patients were selected from an emergency walk-in clinic as being asymptomatic for urological issues; control group 2 (n = 25) has undergone radical prostatectomy with pathologically confirmed organ-confined disease (pT2) and undetectable PSA (≤0.2 ng/mL) at >6 months postprocedure; control group 3 (n = 22), a group with a negative prostate biopsy within 6 months of enrollment and a PSA level ≤10 ng/mL. Full data sets were available for all patients and is summarized in Table 1. All tests were done without knowledge of the grouping of the samples under investigation.

**Sample processing.** Blood samples (15 mL) were collected in the operating room prior to starting any surgical procedure and transported immediately to the Laboratory Medicine, Lahey Clinic. Samples were centrifuged for 10 minutes at 3,000 rpm at room temperature, followed by transfer of plasma to coded tubes. Plasma samples were stored at −70°C and transferred frozen to the Cell and Molecular Biology Laboratory at the beginning of each month. Plasma samples were thawed at room temperature and centrifuged a second time at 3,000 rpm for 10 minutes prior to analysis. A 400x sample was taken from the very top of the centrifuged plasma and DNA was isolated using the Qiagen-DNA Blood Mini Kit (Qiagen, Valencia, CA). The isolated DNA was combined (30:1) with 6 mol/L of guanidine thiocyanate (Sigma, St. Louis, MO) and multiplexed DNA capture probes (Integrated DNA Technologies, Coralville, IA) for sequence-specific DNA fragments in a hybridization-capture technique. Mixes were heated to 95°C for 5 minutes and then incubated at room temperature overnight to facilitate the capture binding of specific DNA fragments. On day 2, the hybridized DNA probe was captured using polystyrene streptavidin beads (Dynabeads M-280 Streptavidin; Invitrogen, Carlsbad, CA). The suspension was rotated for 1 hour at room temperature and the bead complexes were isolated with a magnetic separator and washed four times with washing buffer [1 mol/L NaCl, 10 mmol/L Tris (pH 7.2), 1 mmol/L EDTA, and 1 mL/L of Tween 20]. Following a 1-hour incubation and washing of bead complexes, the captured DNA sequences were released from the Dynabeads with the addition of 48 μL of NaOH (50 mmol/mL). Neutralization buffer (8 μL) was added prior to PCR analysis.

**DNA integrity analysis.** Captured DNA samples were amplified using real-time PCR with a PCR primer set (5 μmol/L, Integrated DNA Technologies), TaqMan probe (2 μmol/L, Integrated DNA Technologies), and the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). Primer sets for the targeted sequences (sequence information can be obtained from Exact Sciences Corporation, Marlborough, MA) were multiplexed in a single reaction for the detection of 200 bp, 1.3, 1.8, and 2.4 kb DNA fragments at a single locus. Four separate loci were analyzed for the presence of the aforementioned DNA fragment lengths. Each TaqMan probe was specific for a primer set and quantitation in genomic equivalents (1 GE = 6.6 pg) was measured via fluorescence throughout the PCR reaction. Positive controls consisted of human genomic DNA standards (0, 20, 100, 500, 2,500, 12,500 GE, Roche Diagnostics, Corp., Indianapolis, IN) in each analytic run. Out of 12 analyzed fragment lengths (excluding 200 bp), 2 loci did not meet the analysis criteria for percentage error and were excluded from analysis. For each of the remaining 10 loci, a cutoff was generated from the Q-PCR data such that the sensitivity and specificity were

### Table 1. Clinicopathologic patient profiles

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Prostate cancer, N (%)</th>
<th>Control group 1, N (%)</th>
<th>Control group 2, N (%)</th>
<th>Control group 3, N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>123</td>
<td>20</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>Age (y), mean ± SD</td>
<td>58.7 ± 6.4</td>
<td>31.6 ± 5.1*</td>
<td>58.5 ± 7.3</td>
<td>61.4 ± 8.5</td>
</tr>
<tr>
<td>PSA (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–4</td>
<td>29 (23.6)</td>
<td>20 (100)</td>
<td>25 (100)</td>
<td>10 (45.5)</td>
</tr>
<tr>
<td>4.1–10</td>
<td>91 (74)</td>
<td>0</td>
<td>0</td>
<td>12 (54.5)</td>
</tr>
<tr>
<td>&gt;10</td>
<td>3 (2.4)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gleason grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>60 (48.8)</td>
<td>N/A</td>
<td>15 (60.0)</td>
<td>N/A</td>
</tr>
<tr>
<td>7 (3 + 4)</td>
<td>44 (35.8)</td>
<td>8 (32.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 (4 + 3)</td>
<td>11 (8.9)</td>
<td>2 (8.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥8</td>
<td>8 (6.5)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor pathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤T2 (confined)</td>
<td>106 (86.2)</td>
<td>N/A</td>
<td>25 (100)</td>
<td>N/A</td>
</tr>
<tr>
<td>&gt;T3 (nonconfined)</td>
<td>17 (13.8)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DIA positive</td>
<td>86 (69.9)</td>
<td>2 (10)</td>
<td>2 (8)</td>
<td>7 (31.8)</td>
</tr>
</tbody>
</table>

*Statistically significant (P < 0.05).

*Adjusted incidence rate after two patients from control group 3 tested positive in a second biopsy and underwent a prostatectomy.
Samples at or above the cutoff were given a score of +1, whereas samples below the cutoff were given a score of 0. The DIA score was generated by summing up the individual scores for each of the 10 loci, thus producing a range from 0 to 10.

**Data analysis.** Each DNA fragment length was assessed independently and a cutoff point was determined to optimize sensitivity and specificity. Categorical data were analyzed using the Pearson \( \chi^2 \) or Fischer’s exact test. The mean and SD were computed for continuous variables and differences between the groups were compared using one-way ANOVA with the Tukey method used for post hoc comparison when variances were equal and Dunnett’s T3 for unequal variances. Two-by-two tables were constructed comparing DIA positivity in those with and without known cancers, and these tables were used to compare sensitivity and specificity. A receiver operating characteristic (ROC) curve was plotted for DIA scores with cancer status as the test variable. All analyses were done using SPSS version 13.0 (SPSS, Inc., Chicago, IL).

**Results**

One hundred and twenty-three patients with prostate cancer and 67 control patients gave their consent to participate in this study to assess the utility of DIA in a plasma-based assay, for the detection of cancer. The characteristics of different patient groups are shown in Table 1. Control group 1 was significantly younger than the other groups, as only men ages <40 were included in this group (\( P < 0.05 \)). PSA values in the cancer group (mean ± SD; 5.3 ± 2.3 ng/mL) were significantly higher than in control group 1 (0.7 ± 0.5 ng/mL) and control group 2 (undetectable range, <0.2; \( P < 0.05 \)) but not compared with control group 3 (4.4 ± 1.5 ng/mL).

Using cell-free DNA isolated from two-spin plasma preparations, we quantitated the amount of DNA associated with different fragment lengths present in patient samples using real-time PCR. Amplification of DNA fragments of 200 bp, 1.3, 1.8, and 2.4 kb were done in a multiplex format for each of the four gene loci evaluated. Figure 1 depicts the amplification scheme used to establish the presence of different DNA fragment sizes for the four different loci. In each case, the 200 bp fragment was used to confirm the presence of DNA validating the sample for further analysis. As stated previously, two DNA fragment lengths did not amplify within the 95% error range for the study and were excluded from the analysis.

Each sample received a DIA score from 0 to 10 in which a DIA cutoff of 7 was selected that maximized the sensitivity and specificity for tumor detection in the overall assay. The data from this analysis is presented as a scatter plot (Fig. 2). The mean DIA scores for the prostate cancer group and control groups 1, 2, and 3 were 7.3 [95% confidence interval (CI), 6.9-7.8], 3.35 (\( P < 0.01 \); 95% CI, 2.0-4.7), 2.8 (\( P < 0.001 \); 95% CI, 2.0-3.6), and 6.1 (\( P = 0.112 \); 95% CI, 5.2-7.1), respectively. A statistically significant difference in mean DIA score was recorded between the prostate cancer group and control groups 1 and 2.
The DIA detected 86 of the 123 prostate cancers evaluated in this study resulting in a sensitivity of 69.9%. In the control groups, a positive DIA score was recorded in 2 of 20 (10%) patients from control group 1, 2 of 25 (8%) patients from control group 2, and 9 of 22 (40.9%) patients from control group 3. Specificity for this assay within the different groups ranged between 59.1% and 92%. Following this determination, two patients from control group 3 who displayed a positive DIA score in the original analysis were identified as having prostate cancer on a repeat prostate biopsy. These findings were identified within 6 months of the original biopsy, resulting in a change in the lower limit of DIA specificity to 68.2%.

ROC curve analysis was used to assess the performance of DIA in identifying tumor presence. As shown in Fig. 3, the area under the ROC curve for DIA was 0.788 (P < 0.001; 95% CI, 0.69–0.86) for determining patients with prostate cancer versus cancer-free patients. The highest sensitivity and specificity were obtained at a DIA score of 7, confirming the optimal cutoff for this assay. Within the prostate cancer group, we compared age, PSA, Gleason score, and tumor pathology in the DIA-positive (≥7) and DIA-negative groups (Table 2). No significant differences were observed. However, all 10 patients ≤49 years of age were positive for DIA. Of the 30 prostate cancer patients with a normal age-adjusted PSA level (age <50, PSA <2.5; age <60, PSA <3.5; age ≥70, PSA <4.5), 19 (63.3%) displayed a DIA score above the cutoff, indicative of tumor presence.

Discussion

The goal of this study was to evaluate the utility of DIA as a plasma-based screening tool to complement PSA in the detection and management of prostate cancer. DIA detected 69.9% of prostate cancer patients and maintained a specificity of 68.2% to 92%, a range comparable to that currently accepted for PSA (60–70%). The considerable range in specificity recorded in this study is largely accounted for by the fact that among the negative biopsy control group 3, some individuals would later be identified as having prostate cancer because transrectal ultrasound biopsy has a 19% to 30% false-negative rate. There exists an inherent bias within this group of patients who were originally enrolled for suspicion of prostate cancer with either an elevated PSA (20 of 22) or a positive digital rectal exam (2 of 22). Indeed, within 6 months of the original sample collection, two patients in this control group were identified as having prostate cancer in a subsequent biopsy. However, at this stage of follow-up, no significant difference between DIA scores from the cancer and negative biopsy groups were recorded. The underlying disease status is more certain in the other control groups, involving males <40 years (control group 1) and postprostatectomy patients (control group 2), in which the combined specificity of DIA was recorded as 91%. Of the two patients scoring >7 in the DIA in control group 1, the one with the maximum score presented to the walk-in clinic with sickle cell anemia, a disease state that may have contributed to the high DIA score recorded. Interestingly, of 30 prostate cancer patients with a normal age-adjusted PSA level, 19 displayed a DIA score indicative of tumor presence. ROC curve analysis was used to assess the performance of DIA in detecting the presence of prostate cancer. The ROC curve showed that DIA has the ability to distinguish the tumor group from the combined control groups in this study in which the area under the curve was 0.788.

There are a growing number of DNA-based assays using cell-free DNA isolated from different bodily fluids probing for...
different variables of change associated with specific disease states (1–17). From such studies, it has become clear that the success and reproducibility of such assays is dependent on a number of factors, most importantly, the collection and processing of patient samples. One recent report has compared the isolation of cell-free DNA from serum and plasma using different preparatory techniques and assessed the levels of the DNA isolated (17). Variations in the sample type and processing techniques were shown to affect the quantity of cell-free DNA rescued. This group reported that two-spin plasma preparations produced the most consistent results, a sample preparatory approach we used in this study. Further considerations in sample preparations have concerned the maintenance of DNA integrity following the isolation of cell-free DNA from spin columns in which shear forces may act to break DNA strands. Such events would undermine the DIA, however, there is little evidence that this is a problem in our study. Other important variables have included time between collection and processing of samples in addition to storage prior to performing the assay, i.e., freeze-thawing of samples and the effects on DNA stability. Differences between collection and processing protocols make it difficult to compare results between groups in which there can be considerable variation in these procedures. It is clear that promising new emergent assays will have to be further evaluated in a prospective setting exercising stringent control over the variables associated with sample collection and processing. The reproducibility of the DIA was confirmed in separate runs involving a subgroup of patients.

Within the prostate cancer arena, there have been a number of studies that have used cell-free DNA isolated from serum, plasma, and ejaculates looking at different variables of change associated with prostate cancer. These include the detection of methylation-specific events centered on specific gene targets, the identification of mutational changes, and quantification of methylation-specific events centered on specific gene targets, associated with prostate cancer. These include the detection of

increase in the quantity of circulating DNA associated with patients with prostate cancer. The source of cell-free DNA is reported to be from cells undergoing apoptosis and necrosis in which such events have been confirmed in in vitro models (19, 29). However, it has recently been hypothesized that cell-free DNA is primarily released by living cells based on observations of increased DNA in cell supernatants from cells in the exponential phase of growth and in normal lymphocyte cultures stimulated with phytohemagglutinin. In both scenarios, there exists few apoptotic or necrotic cells (4).

Results from our study represent a first-step toward the validation of a noninvasive assay with the potential to complement PSA in tumor detection and patient management. Although we have not ascertained whether the DNA integrity index is elevated in the presence of benign prostatic hypertrophy or prostatitis, as is observed with PSA, we have shown that 63% of prostate cancer patients with normal, age-adjusted PSA values, could be identified as cancer-positive using DIA. In this way, DIA can complement PSA in screening for prostate cancer. In an alternative study, an assessment of plasma-based DNA integrity has been done in multiple nonneoplastic states demonstrating the absence of an elevated DNA integrity index associated with nonneoplastic diseases (20). Consistent with our results, the sensitivity and specificity reported for DIA in the detection of female cancers was similar to that reported for the detection of prostate cancer in this study (20). In colorectal and ovarian cancers, alterations in DNA quality rather than quantity have been reported to better characterize tumor-released DNA in different bodily fluids (20, 30). DIA represents a general screen for cancer presence and has yet to be fully tested for changes associated with alternative disease states. The sensitivity and specificity shown for this assay is comparable to many of the current disease markers used in clinical practice (31–33). Clearly, DIA can complement PSA screening by identifying 63% of patients with prostate cancer that present with a normal age-adjusted PSA level. However, larger studies with longer follow-up periods will be needed to establish the utility of DIA in identifying tumor presence in the high-risk group presenting with an indication of prostate disease but a negative biopsy.

Acknowledgments

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