Diagnostic Role of Circulating Free Plasma DNA Detection in Patients With Localized Prostate Cancer

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Abstract

To analyze the potential diagnostic relevance of free plasma DNA (FPDNA), we enrolled 64 patients with localized prostate cancer (CaP). FPDNA was quantified by real-time polymerase chain reaction assessment of the HTERT gene in blood samples from 64 patients with CaP and 45 healthy males. Methylation of the GSTP1 gene was used to confirm the neoplastic origin of FPDNA in selected cases.

The mean ± SD levels of FPDNA were higher in patients with CaP (15.4 ± 10.9 ng/mL) than in control subjects (5.5 ± 3.5 ng/mL; P < .001). By using the best cutoff value, the sensitivity of the test was 80%, the specificity was 82%, the area under the receiver operating characteristic curve, 0.881. High FPDNA values were significantly associated with pathologic T3 stage (P = .035). Methylation of the GSTP1 gene was found in 4 (25%) of 16 FPDNA samples and 15 (94%) of 16 tissue samples.

Quantification of FPDNA discriminates between patients with CaP and healthy subjects and correlates with pathologic tumor stage. FPDNA is a candidate biomarker for early diagnosis and monitoring of CaP.

Prostate cancer (CaP) is the most frequently diagnosed cancer in men in Western countries and the third most frequent malignancy in men in Italy.1,2 The incidence of CaP has dramatically increased in the last 2 decades owing to the extensive application of screening based on serum prostate-specific antigen (PSA) levels.3 Because the PSA test alone is not sufficiently tumor specific, the diagnosis of CaP is made by pathologic evaluation of multiple needle biopsy specimens from patients with suspect serum PSA levels.4 The discovery of biomarkers as tools for early and noninvasive diagnosis of CaP represents a research goal to replace the PSA test.5

The detection of circulating free plasma DNA (FPDNA) seems a promising and noninvasive test for early tumor detection, assessment of recurrence, and therapy control.6-12 Increased levels of FPDNA, compared with levels in healthy subjects, have been observed in patients with several epithelial malignancies, including of the prostate.13-15 The main concern about the application of the FPDNA test in the blood was the occurrence of false-positives, particularly in patients with autoimmune or inflammatory diseases and a recent history of trauma or surgical procedures.8,9 To address this problem, successful characterization of free plasma DNA through searching for specific loss of heterozygosity was recently reported in blood and bone marrow samples from patients with CaP.16,17 The high-throughput quantification of FPDNA and the demonstration of its tumor origin are key points for the application of this test in routine diagnostic procedures.

Promoter hypermethylation is a common epigenetic alteration affecting normal and cancer-related genes. Specific methylation changes are being tested as promising markers for early tumor detection.18 In particular, hypermethylation of the glutathione-S-transferase P1 (GSTP1) is the most common
epigenetic alteration in CaP, and its detection by means of methylation-specific polymerase chain reaction (MSP) discriminates between normal and neoplastic status in prostate tissues and body fluids with high sensitivity and specificity.\textsuperscript{19-23}

We selected 64 patients with early (clinical stage I-II) CaP to assess the diagnostic accuracy of blood FPDNA measured by real-time quantitative polymerase chain reaction (qPCR). Characterization of the neoplastic origin of FPDNA was accomplished by MSP for the GSTP1 gene and controlled in the corresponding CaP tissues after surgery. FPDNA levels were compared with tumor stage and PSA serum levels.

### Materials and Methods

#### Patient Population

We enrolled 64 patients who received a histologic diagnosis of CaP after needle biopsy between November 2003 and June 2004 in our institution. Eligibility criteria for patient selection were as follows: (1) clinical tumor stage I or II, (2) no clinical evidence of lymph node or distant metastases, (3) availability of tumor specimens from radical prostatectomy, (4) availability of blood samples from before surgery, (5) no treatment with hormone or radiation therapy before blood sample collection, and (6) availability of complete clinical, instrumental, and serum PSA data for each patient.

The mean ± SD age of patients with CaP was 64.5 ± 5.9 years \textsuperscript{Table II}. At the time of enrollment, the 64 patients had tumors that were clinical stage I (n = 23 [36%]) or II (n = 41 [64%]). The mean ± SD presurgery PSA level was 9.2 ± 5.5 ng/mL (9.2 ± 5.5 µg/L; range, 1.3-36.2 ng/mL [1.3-36.2 µg/L]). Total serum PSA values were less than 4 ng/mL (4 µg/L) in 2 (3%) and 4 ng/mL (4 µg/L) or more in 62 (97%) patients. After pathologic examination of radical prostatectomy specimens, patients were grouped according to tumor stage as follows: pT2, n = 27 (42%); and pT3, n = 37 (58%). No T4 or N+ patients were encountered. Patients were also grouped according to Gleason score intervals as follows: 1 through 4, n = 4 (6%); 5 or 6, n = 36 (56%); and 7 through 10, n = 24 (38%).

Blood samples from 45 randomly selected healthy male donors were used as negative control samples for the techniques and were collected, extracted, and processed along with and similarly to samples from patients. The mean ± SD total serum PSA level among healthy donors was 1.35 ± 0.76 ng/mL (1.35 ± 0.76 µg/L). In addition, blood samples from 6 patients with benign prostate hyperplasia (BPH) and 2 patients with advanced (disseminated) CaP were also used as control samples for the specificity and sensitivity of the technique.

#### Blood and Tissue Sample Collection

Once enrolled, all patients were scheduled for radical prostatectomy, and 5 mL of blood was drawn just before surgery. Radical prostatectomy specimens were fixed in 4% formalin and entirely submitted for histologic processing. All histologic slides were blindly evaluated by an expert prostate pathologist (M.F.), and pathologic tumor stage was assessed according to the American Joint Committee on Cancer criteria.\textsuperscript{24} Tumor grade was calculated according to the Gleason scoring system.\textsuperscript{25}

The fresh 5-mL blood samples for FPDNA quantification were collected into Vacutainer EDTA tubes (Becton Dickinson, Franklin Lakes, NJ) and processed within 1 hour from collection. In addition, blood samples (3.5 mL) from patients with CaP and BPH were also collected into a Vacuette tube (Becton Dickinson) with serum clot activator beads for serum PSA measurement. Serum PSA measurement was performed using an Advia Centaur immunoassay system (Bayer Healthcare, Tarrytown, NY).

#### FPDNA Quantification

Whole blood from patients and control subjects was centrifuged twice at 2,500g for 10 minutes at 4°C to separate plasma from the cellular fraction. DNA was purified from 1 mL of plasma (QIAamp Blood Mini Kit, Qiagen, Milan, Italy) and eluted in 50 µL of buffer. Quantification of FPDNA was accomplished by real-time qPCR amplification of the human telomerase reverse transcriptase gene (\textit{HTERT}) using the Quantifier Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) on an ABI PRISM 7000 instrument (Applied Biosystems). For each reaction, 2 µL of the

<table>
<thead>
<tr>
<th>Patient Characteristic</th>
<th>Result</th>
<th>Correlation With FPDNA Level</th>
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</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>64.5 ± 5.9</td>
<td>P = .313</td>
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<tr>
<td>Range</td>
<td>50-74</td>
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<tr>
<td>Preoperative PSA level (ng/mL) \textsuperscript{†}</td>
<td>P = .553</td>
<td></td>
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<tr>
<td>Mean ± SD</td>
<td>9.2 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.3-36.2</td>
<td></td>
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<tr>
<td>Clinical tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>23</td>
<td>P = .092</td>
</tr>
<tr>
<td>II</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Pathological tumor stage</td>
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<td></td>
</tr>
<tr>
<td>pT3b</td>
<td>37</td>
<td>P = .035</td>
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<tr>
<td>pT2</td>
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<tr>
<td>Gleason score</td>
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<tr>
<td>1-4</td>
<td>4</td>
<td>P = .153</td>
</tr>
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<td>5-6</td>
<td>36</td>
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<td>7-10</td>
<td>24</td>
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FPDNA, free plasma DNA; PSA, prostate-specific antigen.

\textsuperscript{†} Data are given as number of cases unless otherwise indicated. Mann-Whitney test and Spearman rank correlation used for P value calculations.

\textsuperscript{†} Values are given in conventional units; to convert to Système International units (µg/L), multiply by 1.0.
eluted DNA was used in a final volume of 25 μL and analyzed in duplicate. FPDNA concentrations were calculated by interpolation with the standard amplification curve. qPCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds, and 60°C for 60 seconds for 50 cycles, according to instructions of the manufacturer. Results were expressed as nanograms of DNA per milliliter of plasma.

MSP for GSTP1

To confirm the neoplastic origin of FPDNA, the methylation of the promoter 5′ region of the GSTP1 gene was studied in the blood samples of 16 patients with CaP with FPDNA values more than the cutoff. Of the 16 cases, 10 were in pathologic stage T3 and 6 in T2. Methylation of the GSTP1 gene was also explored in the corresponding paraffin-embedded tissue samples of the primary CaP from the same 16 patients. Neoplastic tissue was isolated by mechanical microdissection under a Nikon inverted microscope (Eclipse TE2000, Nikon, Tokyo, Japan). Blood samples from 16 healthy blood donors, the 6 patients with BPH, and the 2 patients with disseminated CaP were also tested for GSTP1 methylation as further control experiments for the specificity and sensitivity of the technique. The prostate cancer cell line LNCaP, which is reported to harbor homozygous methylation of GSTP1, was used as a positive control sample for the technique and was cultured in RPMI 1640 medium (Gibco, Paisley, Scotland) supplemented with 10% fetal bovine serum (Gibco). To assess the sensitivity of MSP for GSTP1, total DNA from the LNCaP cell line was serially diluted from 1 to 0.001 μg.

DNA extraction from paraffin-embedded tissue samples was performed using the QiAamp Mini Kit (Qiagen). For MSP, 0.1 μg of DNA extracted from tissue and 0.005 to 0.1 μg of DNA extracted from plasma were used. Bisulfite modification of FPDNA was accomplished by using the CpGenome DNA Modification Kit (Qbiogene, Montreal, Canada). MSP was carried out using specific fluorescently labeled primers for methylated (forward, 5′-6FAM-TTC GGG GTG TAG CGG TCG TC-3′; reverse, 5′-GCC CCA ATA CTA AAT CAC GAC G-3′ [92 base pairs (bp)]) and unmethylated (forward, 5′-HEX-GAT GTT GGG GTG TAG GTT GGC AGC C-3′; reverse, 5′-CCA CCC CAA TAC TAA ATC ACA ACA-3′ [99 bp]) GSTP1.18 MSP was performed with a hot-start procedure (HotStart Taq polymerase, Qiagen) at the following conditions: 95°C for 15 minutes, 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds for 55 cycles with final extension for 8 minutes at 72°C. Fluorescent MSP products were separated electrophoretically on an 8% acrylamide gel and visualized under a UV transilluminator after staining with ethidium bromide. Selected MSP products were analyzed by laser fluorescence using an automated gene sequencer (ABI Prism 37XL DNA sequencer, Applied Biosystems) and GeneMapper ID software, version 3.2 (Applied Biosystems).

Statistical Analysis

Data are reported as mean ± SD, range, and frequency. The Mann-Whitney test and the Spearman rank correlation were applied. The receiver operating characteristic (ROC) curve and the respective area under the curve (AUC) were calculated for FPDNA to provide more accurate information to distinguish patients with CaP from healthy control subjects.26 Nonparametric estimates of the area under the ROC (AUC ROC) curves and the respective SEs were applied.27 The best cutoff values were chosen as the values that maximized the likelihood ratio obtained by using the following formula: Likelihood Ratio = (Probability of True-Positive + Probability of True-Negative)/(Probability of False-Positive + Probability of False-Negative). The best cutoff has been reported as the range in which the diagnostic performance of the method did not change.28 By using this cutoff value, the sensitivity and the specificity were calculated.

Data analysis was performed using SPSS for Windows (version 13.0, SPSS, Chicago, IL). A 2-tailed P value less than .05 was used to define statistical significance.

Ethics

The study was approved by the ethical committee of the S. Orsola-Malpighi Hospital, Bologna, Italy. Informed consent was obtained from all patients by signature on the specific form provided by the ethical committee. The study protocol conformed to the ethical guidelines of the “World Medical Association Declaration of Helsinki—Ethical Principles for Medical Research Involving Human Subjects” adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo, Japan, 2004.

Results

FPDNA Quantification Discriminates Between Patients With CaP and Healthy Control Subjects

The mean ± SD levels of FPDNA were significantly higher in patients with CaP (15.4 ± 10.9 ng/mL; range, 4.2-67.1) than in healthy control subjects (5.5 ± 3.5 ng/mL; range, 1.0-15.2 ng/mL; P < .001; Mann-Whitney test) (see clustering Figure 1). The best cutoff value to discriminate between patients and healthy control subjects was between 7.95 and 8.08 ng/mL and for practical purposes was fixed at 8 ng/mL. The sensitivity of the test was 80% (51/64 patients with CaP with FPDNA values more than the cutoff). The specificity was 82% (37/45 healthy control subjects with FPDNA values less than the cutoff). The AUC ROC curve was 0.881 ± 0.032 (Figure 2).

The FPDNA values for the 2 patients with disseminated CaP were more than the cutoff (ie, 15.9 and 281.5 ng/mL).

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Among the 6 patients with BPH, 1 (17%) had an FPDNA value more than the cutoff (32.2 ng/mL), and values for the remaining 5 were less than the cutoff (mean, 4.5 ng/mL).

Pathologic Tumor Stage Correlates With FPDNA Levels

Significantly higher FPDNA values were found in patients with pathologic T3 tumor stage compared with T2 (17.5 ± 12.1 vs 12.6 ± 8.4 ng/mL; P = .035; Mann-Whitney test). By contrast, the correlation between FPDNA levels in clinical stage I and II did not reach significance (17.0 ± 12.0 vs 12.7 ± 8.1 ng/mL; P = .092; Mann-Whitney test). No statistical associations were found between FPDNA and age, Gleason score, and total serum PSA level before surgery (P = .313, P = .153, and P = .553, respectively; Spearman rank correlation). No significant statistical association was found between PSA level and pathologic stage or clinical tumor stage (P = .308 and P = .834, respectively; Mann-Whitney test).

GSTP1 Methylation Characterizes FPDNA

Methylation of the promoter region of the GSTP1 gene was revealed by MSP in 4 (25%) of 16 plasma samples from patients with CaP and in 2 (100%) of 2 samples from patients with disseminated CaP. MSP performed on tumor DNA extracted from the corresponding CaP paraffin-embedded, microdissected tissue samples from the same patients showed that GSTP1 was methylated in 15 (94%) of 16 cases, including the 4 cases that were methylated in the plasma (100% concordance). GSTP1 methylation was also demonstrated in 2 needle biopsy specimens from the patients with disseminated CaP. The DNA extracted from the LNCaP cell line always showed GSTP1 methylation as expected and was used as the positive control sample in each run of MSP (Figure 3). Sequence analysis of the MSP fluorescent products confirmed the presence of methylated (92-bp) and unmethylated (99-bp) GSTP1 (Figure 3). None of the FPDNA samples from the 16 healthy control subjects showed GSTP1 methylation. Of the 6 FPDNA samples from patients with BPH, 1 (17%) showed methylation of GSTP1.

Discussion

Serum PSA measurement is the standard procedure for diagnostic screening and assessment of biochemical recurrence of CaP in men. The qualifications of a novel CaP diagnostic marker able to replace serum PSA level as the marker in the future include the following: (1) higher sensitivity and specificity; (2) closer relation to clinical and pathologic tumor features; (3) comparable ease of technical feasibility; and (4) good cost/benefit ratio. The detection of circulating nucleic acids and circulating tumor cells represents a cutting edge for the research into new diagnostic and prognostic biomarkers.

We describe the ability of total FPDNA quantification to effectively (P < .001) discriminate between patients with CaP and healthy male control subjects. The sensitivity (80%) and specificity (82%) of our test and the AUC ROC value (0.881) that we obtained can be considered good for a blood diagnostic test. In addition, the 2 patients with disseminated CaP showed high levels of FPDNA, whereas all but 1 of the patients with BPH had FPDNA values less than the cutoff.
The 1 patient with BPH with a positive result also had relevant clinical symptoms of acute prostatitis, suggesting a possible FPDNA elevation owing to this inflammatory disorder. Many nonneoplastic pathologic conditions such as autoimmune disorders, inflammatory diseases, and trauma are associated with elevations of FPDNA in the blood.\(^8,9\) These factors might also explain the FPDNA levels more than the cutoff detected in some healthy subjects. Similarly, the FPDNA
values did not reach the cutoff in a portion of patients with CaP. We think this latter finding is not related to a low DNA amplification capability of our real-time PCR method, but rather might be ascribed to a defect of free DNA release from some tumors. In fact, regarding the origin of circulating tumor nucleic acids, it remains unclear whether free DNA is just released, is actively secreted by the primary tumor, or derives from the rupture of circulating neoplastic cells.

A further confirmation of the specificity of our test was obtained with the characterization of FPDNA by search for the methylation of the GSTP1 gene. In fact, all healthy blood donors and all but 1 of the patients with BPH did not have GSTP1 methylation in the plasma. The detection rate of GSTP1 methylation in our series was 25% in the plasma and 94% in the corresponding paraffin-embedded CaP tissue samples. Methylation in the promoter region of the GSTP1 gene is an early epigenetic alteration in CaP, and it is detected in approximately 90% of CaP tissue samples.19,21 In the plasma of patients with CaP, the detection rates of GSTP1 methylation range between 13% and 72% according to different reports (see review by Henrique and Jeronimo19). This variability has been explained by the enrollment of different populations of patients with CaP (which also included subjects with disseminated neoplastic disease) or by the use of a quantitative rather than a conventional MSP procedure. We decided to use a conventional MSP approach because it seems more sensitive in plasma and there is no current evidence that the quantitative MSP is more specific.23 Furthermore, our GSTP1 methylation detection rate in the plasma (25%) was consistent with the data from another report on patients with low-stage CaP without evidence of tumor dissemination.23 The presence of plasma GSTP1 methylation in the patient with BPH is also not surprising because it was previously demonstrated in normal prostate tissues.23 In addition, we cannot exclude that this patient might harbor an occult microscopic focus of CaP in the context of BPH.

One of the main findings of our study regards the significant statistical association (P < .05) between high values of FPDNA and pathologic T3 tumor stage in the corresponding radical prostatectomy specimens. This represents one of the first demonstrations of clinical relevance of FPDNA quantification in CaP. Similar associations have been observed in other epithelial malignancies, suggesting the neoplastic origin of FPDNA. The explanation of the mechanism of tumor DNA release in the blood of patients with CaP is unknown and beyond the scope of the present study. We cannot claim that the FPDNA found in patients with stage pT3 CaP derives from extraprostatic cancer cells. FPDNA might be released from the tumor burden or from circulating tumor cells. As a matter of fact, FPDNA levels in patients with stage pT2 were significantly lower compared with levels in patients with stage pT3. This finding suggests that FPDNA testing might represent a tool for molecular staging of CaP that is more valuable than serum PSA testing. In fact, no correlation between serum PSA and stage was found in our series.

On technical grounds, we demonstrated that our real-time PCR-based FPDNA quantification is a quick, easy, noninvasive, and highly reproducible method. It is also our opinion that the costs of the real-time PCR instrument can be at least partially covered by the high throughput of the technique. The data generated by us in the present study and by other laboratories in recent studies suggest that the FPDNA quantification test could represent a promising candidate biomarker for the early diagnosis of CaP and the monitoring of recurrence.

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