**DD3PCA3**, a Very Sensitive and Specific Marker to Detect Prostate Tumors

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**ABSTRACT**

We identified DD3PCA3 as one of the most prostate cancer-specific genes at present (M. J. Bussemakers et al. Cancer Res., 59: 5975–5979, 1999). Consequently, DD3PCA3 is an interesting candidate for use as a diagnostic and/or prognostic marker. In this study we developed a method for the accurate quantification of DD3PCA3 mRNA, using real-time quantitative reverse transcription-PCR. DD3PCA3 was expressed at low levels in normal prostate but not in 21 selected other normal tissues, blood, or 39 tumor samples other than prostate. The diagnostic and prognostic value of DD3PCA3 in normal, hyperplastic, and malignant prostate tissues was determined and compared with another promising tumor marker for prostate cancer, telomerase reverse transcriptase (hTERT gene), the expression of which is related to telomerase activity. Sensitivity and specificity estimates for both genes were calculated as the area under the receiver-operating characteristics curve (AUC-ROC). DD3PCA3 (AUC-ROC, 0.98) demonstrated better diagnostic efficacy than hTERT (AUC-ROC, 0.88). Moreover, the median increase in mRNA expression in tumor tissues compared with nonmalignant prostate tissues was much higher for DD3PCA3 (34-fold) than for hTERT (6-fold). In tumor tissues, the median expression of DD3PCA3 was much higher than hTERT (5840 versus 10 normalized mRNA copies). A significant relationship was observed only between tumor stage and hTERT gene expression. We conclude that expression of the DD3PCA3 gene is a very sensitive and specific marker for the detection of prostate tumor cells in a high background of normal (prostate) cells. Consequently, DD3 measurements may be used for clinical application in prostate needle biopsies or bodily fluids such as blood, ejaculate, urine, or prostate massage fluid.

**INTRODUCTION**

Prostate cancer is the most frequently diagnosed cancer in the Western male population and the second leading cause of cancer mortality. Patients with organ-confined prostate cancer are generally treated with radical surgery or radiation therapy. Alternatively, if the tumor has spread locally or distantly, androgen ablation is the standard therapy used. Unfortunately, most of the latter patients will develop progressive disease, and there is no other effective treatment available. Therefore, methods are necessary that can detect the tumor early, at a time when the tumor is still locally confined and potentially curable by radical prostatectomy.

A significant contribution to the early detection of prostate cancer has been the discovery of PSA and subsequent development of various immunological assays in serum. Serum PSA is now recognized as the premier marker for detection of prostate cancer and can be used for screening selected populations of patients and for monitoring patients after therapy (1). However, serum PSA levels are regularly elevated in men with BPH, prostatitis, and other nonmalignant disorders, resulting in reduced specificity (1).

Novel cancer-specific markers have been identified that may aid early diagnosis and help to differentiate between tumor and nonmalignant growth. Telomerase activity is one of the most promising markers. High activity has been detected in the majority (90%) of prostate tumors (2, 3), whereas only low or absent activity was observed in normal and BPH tissues (3–5). An alternative for telomerase activity measurement is the quantification of telomerase reverse transcriptase mRNA (hTERT gene) by use of real-time quantitative PCR (6). Because hTERT expression is the rate-limiting determinant of the telomerase enzyme (7), accurate quantification of hTERT mRNA copies may better differentiate between malignant and benign prostate growth than semiquantitative telomerase activity measurements.

Next to hTERT gene expression, other genes, such as PSGR and PGCGEM1, have recently been identified that have more prostate-specific expression (8, 9). We identified and characterized DD3PCA3, a new prostate-specific gene (10). Northern blot analysis showed that DD3 mRNA is expressed at low levels in normal prostate and is abundantly present in prostate tumor tissues (10). A more quantitative, reproducible, and sensitive assay is necessary to test DD3 as a diagnostic or prognostic marker in clinical samples, which often contain only small amounts of mRNA.

**MATERIALS AND METHODS**

**Tissues.** Normal tissue samples were obtained from autopsies with a postmortem delay of <4 h. These tissues, together with 39 frozen tissues from nine different types of tumors (lung, esophagus, ileum, colon, pancreas, testis, breast, bladder, and melanoma), were randomly selected from the tissue bank of the Department of Pathology (University Medical Centre Nijmegen). Prostate tissue sections (normal, BPH, and tumor) were carefully selected and removed by a pathologist from fresh prostate of patients after radical prostatectomy at the Canisius-Wilhelmina Hospital (Nijmegen, the Netherlands) and the University Medical Centre Nijmegen. The stages (TNM) of these tumors were determined at the Department of Pathology of both hospitals. Frozen tumor, BPH, and normal prostate tissues were step-sectioned with evaluation by H&E staining at regular intervals. This evaluation included determination of percentage of normal, BPH, and tumor cells, together with Gleason score of the tumor sections.

**RNA Isolation and cDNA Synthesis.** Total RNA from healthy tumor and tumor tissues was isolated by disruption of 10–25 frozen, 20-μm sections in 1 ml of R.NaO/1B (Biotex Laboratories Inc., Houston, TX) or TRIzol (Life Technologies, Inc., Breda, the Netherlands) using a sterile pestle. After the manufacturer’s protocols were completed, RNA was further purified using the RNasy kit (Qiagen, Hilden, Germany). RNA was quantified spectrophotometrically.

Purified RNA (0.2–1.0 μg) was added to RNase-free water to a final volume of 10 μl, denatured for 5 min at 90°C, and cooled immediately on ice. Reverse transcription mixture (10 μl) was added, containing first strand buffer (Life Technologies, Inc.), 20 units of Rnasin (Promega, Madison Wisconsin), 10 mM DTT, 4.75 mM random hexamers, and 600 μM deoxynucleotides. After annealing of the hexanucleotides for 10 min at 20°C, cDNA synthesis was performed for 45 min at 42°C, followed by an enzyme inactivation step for 5 min at 95°C. cDNA was stored at −20°C until use.

**Quantitative Measurement of PCA3, hTERT, and 18S rRNA.** The gene sequence of DD3 is presented in GenBank accession no. AF103907. The structure of the DD3 gene with exon-intron boundaries was described previously (10).
The expression of DD3 in normal tissues was characterized as follows:

- **Skin**: rRNA = 4.8 × 10^6, DD3 = 0, Normalized DD3 = 0
- **Skeletal muscle**: rRNA = 48.0, DD3 = 0, Normalized DD3 = 0
- **Heart**: rRNA = 12.0, DD3 = 0, Normalized DD3 = 0
- **Lymph gland**: rRNA = 26.0, DD3 = 0, Normalized DD3 = 0
- **Spleen**: rRNA = 38.0, DD3 = 0, Normalized DD3 = 0
- **Bone marrow**: rRNA = 4.6, DD3 = 0, Normalized DD3 = 0
- **Lung**: rRNA = 12.0, DD3 = 0, Normalized DD3 = 0
- **Stomach**: rRNA = 20.0, DD3 = 0, Normalized DD3 = 0
- **Ileum**: rRNA = 13.0, DD3 = 0, Normalized DD3 = 0
- **Colon**: rRNA = 16.0, DD3 = 0, Normalized DD3 = 0
- **Liver**: rRNA = 40.0, DD3 = 0, Normalized DD3 = 0
- **Pancreas**: rRNA = 24.0, DD3 = 0, Normalized DD3 = 0
- **Testis**: rRNA = 28.0, DD3 = 0, Normalized DD3 = 0
- **Bladder**: rRNA = 34.0, DD3 = 0, Normalized DD3 = 0
- **Kidney**: rRNA = 52.0, DD3 = 0, Normalized DD3 = 0
- **Uterus**: rRNA = 44.0, DD3 = 0, Normalized DD3 = 0
- **Thyroid gland**: rRNA = 2.8, DD3 = 0, Normalized DD3 = 0
- **Brain**: rRNA = 14.0, DD3 = 0, Normalized DD3 = 0
- **Esophagus**: rRNA = 7.2, DD3 = 0, Normalized DD3 = 0
- **Ovaries**: rRNA = 7.8, DD3 = 0, Normalized DD3 = 0
- **Breast**: rRNA = 0.6, DD3 = 0, Normalized DD3 = 0
- **Prostate**: rRNA = 4.6, DD3 = 699, Normalized DD3 = 152
- **Leukocytes**: rRNA = 34.0, DD3 = 0, Normalized DD3 = 0

To visualize the efficacy of the two markers to discriminate tumor tissue from normal tissue (in the absence of an arbitrary cutoff value), we summarized the data in a ROC curve. This curve plots the sensitivity (true positives) on the Y axis against 1 — the specificity (false positives) on the X axis, considering each observed value as a possible cutoff value. The AUC was calculated as a single measure for the discriminatory efficacy of a marker. When a marker has no discriminatory value, the ROC curve will lie close to the diagonal and the AUC is close to 0.5. When a test has strong discriminatory value, the ROC curve will move up to the upper left-hand corner and the AUC will be close to 1.0. The Statistical Package for Social Sciences (SPSS) was used for analyses.

### RESULTS

**Prostate Specificity of DD3.** Absolute copy numbers of DD3 mRNA were determined from cDNA from 22 different normal tissues and peripheral blood leukocytes. These absolute copy numbers were normalized to the expression of rRNA (Table 1). Normal prostate tissue expressed DD3 mRNA at a low level. DD3 was absent in all other tissues, except for kidney, in which DD3 was expressed at an insignificant level.

In addition, DD3 mRNA expression was measured in 39 tumor samples representing nine different tumor types (Table 2). DD3 mRNA could not be detected in all tested tumor samples. The absence of expression of DD3 in the vast majority of both normal and tumor tissues other than prostate demonstrated the high prostate specificity of this novel tumor marker.

**Diagnostic Performance of hTERT and DD3 as Prostate Tumor Markers.** hTERT and DD3 mRNA expression was quantitatively measured in 11 normal prostate tissues, 5 BPH samples, and 31 prostate adenocarcinomas (Table 3). No significant differences in hTERT or DD3 mRNA expression were detected between normal and BPH tissues. Therefore, normal and BPH tissues together were defined as one group of nonmalignant tissues.

Median hTERT mRNA expression levels between nonmalignant (median, 1.7; range, 0–5.2) and tumor tissues (median, 10.1; range, 0.7–76.1) were significantly different (P < 0.0001). Median up-regulation of hTERT expression from normal to tumor tissues was 6-fold. A significant relationship was found between hTERT expression and tumor stage (P = 0.02), but not with Gleason score.

For DD3, median normalized expression was also significantly different between nonmalignant (median, 174; range, 0–593) and malignant prostate tissues (median, 5,849; range, 334–39,456; P < 0.0001). Median up-regulation of DD3 from normal to tumor tissues was 34-fold. Even in tissues containing ≤10% tumor cells, normalized DD3 expression was clearly higher than that in nonmalignant tissues (Table 3, samples 38–47). No significant correlation was found between DD3 expression and tumor stage or Gleason score.

A ROC curve was constructed for both markers (Fig. 1). The AUC-ROC represents the diagnostic efficacy of the continuous test result. The AUC-ROC was 0.98 (95% confidence interval, 0.96–1.00) for DD3 and 0.88 (95% confidence interval, 0.78–0.97) for hTERT, indicating good discrimination power for both tests.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>rRNA* (×10^6)</th>
<th>DD3*</th>
<th>Normalized DD3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin</td>
<td>4.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>48.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heart</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymph gland</td>
<td>26.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>38.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>4.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>12.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stomach</td>
<td>20.0</td>
<td>0</td>
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</tr>
<tr>
<td>Ileum</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colon</td>
<td>16.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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</tr>
<tr>
<td>Pancreas</td>
<td>24.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Testis</td>
<td>28.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bladder</td>
<td>34.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Kidney</td>
<td>52.0</td>
<td>390</td>
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<tr>
<td>Uterus</td>
<td>44.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>2.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>14.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Esophagus</td>
<td>7.2</td>
<td>0</td>
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</tr>
<tr>
<td>Ovaries</td>
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</tr>
<tr>
<td>Breast</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Prostate</td>
<td>4.6</td>
<td>699</td>
<td>152</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>34.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ct values were transformed to absolute gene copy numbers by calibration curves (5-log range) of rRNA and DD3 plasmid calibrators. The functions of the calibration curves were: f(Ct) = −3.40 log [rRNA copies/20] + 40.80; and f(Ct,DD3) = −3.84 log [DD3 copies] + 45.32.

**Table 2 Normalized DD3 expression in pooled tumor tissues**

<table>
<thead>
<tr>
<th>Tumor pool</th>
<th>n</th>
<th>rRNA* (×10^6)</th>
<th>DD3</th>
<th>Normalized DD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>5</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Esophagus</td>
<td>6</td>
<td>7.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Colon</td>
<td>4</td>
<td>15.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bladder</td>
<td>5</td>
<td>13.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Melanoma</td>
<td>3</td>
<td>8.8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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DISCUSSION

We developed a method for the accurate quantification of \( \text{DD3} \) mRNA expression and showed that \( \text{DD3} \) was exclusively expressed in the prostate. For diagnosis of tumor, \( \text{DD3} \) expression was compared with the expression of \( h\text{TERT} \) mRNA. \( h\text{TERT} \) gene expression is the rate-limiting determinant of telomerase activity (7) and at present the most common determinant of telomerase activity (7) and at present the most common diagnostic characteristic (13, 14). The median increase in \( h\text{TERT} \) mRNA expression from nonmalignant to malignant tissues was only 6-fold for \( h\text{TERT} \), whereas the median \( \text{DD3} \) increase was 34-fold. A large increase is important for the detection of a few malignant cells in a high background of normal prostate cells. This was clearly shown for 10 tissue samples containing \( \leq 10\% \) tumor cells, in which \( \text{DD3} \) showed higher sensitivity than \( h\text{TERT} \). Moreover, in prostate tumors, the median normalized expression of \( \text{DD3} \) mRNA was \( \sim 580\)-fold higher than \( h\text{TERT} \) mRNA (5849 versus 10 normalized copies, respectively). This high expression in tumor cells may also be advantageous for the detection of the few malignant prostate cells shed into blood, urine, prostatic massage fluid, or ejaculate. In addition, \( \text{DD3} \) mRNA expression could not be detected in leukocytes, which are regularly present in these bodily fluids, whereas \( h\text{TERT} \) mRNA is expressed in leukocytes and may cause false positivity (13, 14).

We previously detected a statistically significant relationship between the expression of \( h\text{TERT} \) mRNA and tumor stage and grade in urothelial cell carcinomas (15). Others found a similar relationship for breast tumors and Wilms’ tumors (16, 17). In the present study in prostate tumors, this correlation was observed only for stage, but not for Gleason score.
(grade). The 21 analyzed prostate tissues (with tumor cells ≥20%) were derived from radical prostatectomies, with only little variation in stage and grade. This low number may have caused the lack of correlation with grade. In contrast, Latil et al. (18) did not detect a correlation between hTERT expression and pathological parameters for 33 prostate tumor samples with a broader range of stage and Gleason grade.

Typically, both we (present study) and Latil et al. (18) detected low hTERT expression in the majority of samples from normal prostate tissues and BPH. This contrasts with findings using the telomeric repeat amplification protocol, with which telomerase activity could not be detected in most normal and BPH tissues (3–5). The difference can be explained by the increased sensitivity of the real-time reverse transcription-PCR assay (16, 18). Moreover, low hTERT expression in normal prostate tissues corroborates findings that prostatic tissues contain stem cells (19), which generally express telomerase activity.

Recently, DD3 has been described as one of the most prostate cancer-specific genes (10). Unfortunately, the biological function of DD3 has not been unraveled, and no homology to any gene present in the computer databases has been found (10). Because of the lack of extensive open reading frames, we suspect that the gene functions as a noncoding RNA (10). Therefore, a mRNA-based method was necessary to quantify the expression of DD3 in tissues. Our quantitative gene expression data showed that DD3 is regulated by a unique prostate cancer-specific transcriptional mechanism. However, no correlation between transcriptional activity and tumor stage or grade was detected. For future clinical applications, our real-time, quantitative reverse transcription-PCR test will provide a very sensitive and specific tool to detect prostate tumor cells in tissue biopsies and bodily fluids.

REFERENCES


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