Plasma Levels of Trefoil Factors are Increased in Patients with Advanced Prostate Cancer

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Abstract

Purpose: Through cDNA array analyses and immunohistochemistry on tissue microarrays, trefoil factor 3 (TFF3) was recently shown to be overexpressed in prostate cancer. The purpose of this study was to test the feasibility of using the levels of trefoil factors as a plasma marker for prostate cancer.

Experimental Design: In 79 patients with prostate cancer, 23 patients with benign prostatic hyperplasia, and 44 healthy individuals plasma TFF1, TFF2, and TFF3 were determined with ELISAs and compared with clinical stage and prostate-specific antigen (PSA) values. Plasma levels of TFF were compared with the immunohistochemical expression of TFF and chromogranin A in 30 prostate cancer tissue samples.

Results: Patients with advanced prostate cancer had significantly higher plasma concentrations of TFF1, TFF2, and TFF3 (P < 0.01) compared with patients with localized disease. Using a cutoff of 200 pmol/L, the sensitivity and specificity of plasma TFF3 in differentiating between patients with localized and advanced disease was 74% (59–86%) and 81% (66–91%). Plasma levels of TFF3 were highest in patients with bone metastases (P = 0.008). Patients with serum PSA >10 µg/L had significantly higher plasma TFF3 values than patients with serum PSA <10 µg/L (P = 0.03) and TFF3 levels were higher in patients with Gleason sums of ≥7 (P = 0.02). Expression of TFF1 and TFF3 determined by immunohistochemistry was increased in patients with prostate cancer but did not correlate with plasma trefoil factor values.

Conclusions: Plasma levels of trefoil factors are increased in patients with advanced prostate cancer. Prospective studies are needed to confirm the predictive utility of trefoil factors in prostate cancer.

Additional biomarkers are needed in prostate cancer that could increase the specificity and sensitivity of prostate cancer diagnosis and predict the rate of progression of prostate cancer as well as the response to particular interventions (1). Through cDNA array analyses and immunohistochemistry on prostate cancer tissue arrays, trefoil factor 3 (TFF3) was recently shown to be overexpressed in prostate cancer (2–6). Previously, TFF1 was also shown by immunohistochemical and mRNA analysis to be differentially expressed in the human prostate and prostate cancer (7, 8).

The trefoil factors (TFF1-3) constitute a family of small peptides that are associated and cosecreted with mucins in mucin-secreting epithelial cells and play a critical role in mucosal protection and repair (9). The peptides have been named trefoil factors due to the existence of a common three-loop structure, which makes the peptides extremely stable toward proteolytic digestion, acid, and heat degradation. The mammalian trefoil factor family includes TFF1 (breast cancer–associated peptide or PS2; refs. 10, 11), TFF2 (spasmolytic polypeptide; refs. 12–14), and TFF3 (intestinal trefoil factor; refs. 15, 16). Members of the trefoil peptide family have been observed to be overexpressed in a variety of cancers, including intestinal, pancreas, and prostate cancers (6, 8, 17–19) and function as scatter factors, proinvasive, antiapoptotic, and angiogenic agents (17, 20–23).

We previously developed and validated ELISA assays for the quantification of human TFF1, TFF2, and TFF3 in biological fluids (24, 25). The purpose of the present cross-sectional study was to examine the levels of trefoil factors in plasma of patients with prostate cancer and in controls, and to correlate with clinicopathologic variables in order to investigate the potential value of quantification of plasma trefoil factors in the diagnosis of prostate cancer.

Materials and Methods

Plasma samples. TFF1-3 was measured in plasma samples from 23 patients with histologically confirmed benign prostatic hyperplasia...
(BPH) and 79 patients with prostate cancer treated at the Department of Urology at Aarhus University Hospital from the period 1994 to 2004. The patients with prostate cancer were classified into three subgroups as follows: 27 patients with newly diagnosed untreated advanced disease, 15 previously castrated patients with hormone refractory advanced disease, 37 patients with localized disease \( (T_1, T_2, N_0, M_0) \) who underwent radical prostatectomy. Diagnoses of BPH were histopathologically confirmed using tissue material obtained by transurethral resection. In seven BPH patients with significantly increased prostate-specific antigen (PSA) values, the diagnoses were also confirmed by transrectal ultrasound-guided prostate biopsy. Patients with renal dysfunction (creatinine >133 \( \mu \text{mol/L} \)) were excluded (seven patients). The control group consisted of 44 presumably healthy male blood donors (ages 50-64 years; median, 57 years; Table 1).

Archived (at \(-80^\circ\)C) plasma samples collected in the period 1994 to 2004 were used. All plasma samples were taken before any diagnostic procedure. The local scientific ethics committee approved all procedures. All participants gave written and oral consent.

**Immunohistochemistry.** To establish expression patterns of TFF1, TFF2, and TFF3 and differences in pattern of expression from that of chromogranin A, immunostaining of these substances was done in prostate specimens from 30 patients: 8 patients with advanced prostate cancer, and 10 patients undergoing transurethral resection of the prostate for benign glandular and fibromuscular hyperplasia. The immunohistochemical staining was done on 5-\( \mu \)m sections of formalin-fixed, paraffin-embedded tissue samples.

For immunostaining of TFF1, TFF2, and TFF3, the sections were incubated for 30 minutes in 10\% normal rabbit serum (Dako, Glostrup, Denmark), and then for 18 hours at room temperature with the primary antibody polyclonal rabbit anti-human chromogranin A (Dako) diluted 1:400. The immunoreactions for TFF1, TFF2, and TFF3 were visualized by means of biotinylated swine anti-rabbit immunoglobulins (Dako) diluted 1:40 for 1 hour as the second layer followed by StreptABComplex/horseradish peroxidase (Dako) diluted 1:100 as the third layer, and finally stained by means of 3,3-diaminobenzidine for 30 minutes. The immunoreactions for chromogranin A were visualized by Dako EnVision+ System (Dako). The sections were counterstained with hematoxylin.

**Molecular sieve chromatography.** Plasma samples (350-500 \( \mu \)L) from four patients with prostate cancer and two patients with BPH were subjected to size-exclusion chromatography on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech) using a SMART system (Amersham Pharmacia Biotech). The column was pre-equilibrated and eluted with 0.05 mol/L Tris-HCl (pH 7.2), 0.3 mol/L NaCl containing 1 g/L human albumin, and 0.2 g/L sodium azide at 0.5 mL/min with collection of 0.5 mL fractions. We determined the distribution of TFF3 immunoreactivity by TFF3-ELISA.

**Statistical analysis.** We used the Analyse-it version 1.69 add-in program package for Microsoft Excel (Analyse-it Software, Ltd., Leeds, United Kingdom) for statistical analysis. We analyzed differences between the median of groups by the Kruskal-Wallis one-way ANOVA by ranks and the Mann-Whitney \( U \) test. Spearman rank correlation was used to compare the variables of PSA, plasma trefoil factors, and Gleason score. Parametric reference intervals were used and computed as described (26). Due to nonnormal distribution of the reference values, all observations were analyzed on a logarithmic scale. Confidence intervals for sensitivity, specificity, positive predictive value, and negative predictive value were calculated according to (27). \( P < 0.05 \) was considered statistically significant in a two-sided test.

**Results**

**Plasma levels of trefoil factors.** In Table 1, median concentrations of the TFFs are given for patients with prostate cancer, patients with BPH, and healthy controls. On the basis of TFF measurements on 44 healthy male individuals >50 years of age, we defined new age- and sex-specific parametric reference intervals for plasma measurements of trefoil peptides to be 220 to 1,400 pmol/L, 67 to 210 pmol/L, and 74 to 200 pmol/L for TFF1, TFF2, and TFF3, respectively.

Figure 1 shows the distributions of TFF for the four patient groups and the healthy individuals. The TFF concentrations of

**Table 1.** Patient characteristics and blood markers

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>( n )</th>
<th>Age, median (range)</th>
<th>Gleason score</th>
<th>PSA (( \mu )g/L)</th>
<th>Plasma trefoil peptides</th>
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<tbody>
<tr>
<td></td>
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<td>2-6</td>
<td>7-10</td>
<td>TFF1, pmol/L</td>
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<td>TFF3, pmol/L</td>
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<td>median (range)</td>
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<td>BPH</td>
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<td>66 (53-79)</td>
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<td>8</td>
<td>2</td>
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<td>27</td>
<td>78 (53-91)</td>
<td>0</td>
<td>1</td>
<td>26</td>
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<td>65 (51-74)</td>
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<td>15</td>
<td>73 (50-78)</td>
<td>4</td>
<td>0</td>
<td>11</td>
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<td>Healthy individuals</td>
<td>44</td>
<td>57 (50-64)</td>
<td>520</td>
<td>2</td>
<td>120 (57-220)</td>
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*There was no information on the Gleason score in one patient.
†There was no information on the Gleason score in four patients.
the healthy men and BPH patients differed significantly for TFF1 and TFF3. Patients with prostate cancer with localized disease had significantly higher plasma concentrations of TFF3 than healthy controls. No difference was observed between patients with BPH and patients with prostate cancer with localized disease.

There was no difference between TFF levels in plasma from untreated and hormone refractory prostate cancer patients with advanced disease. Both these subgroups had significantly higher plasma concentrations of TFF1, TFF2, and TFF3 (P < 0.01) compared to patients with prostate cancer with localized disease. For TFF2 and TFF1, the groups generally showed much overlap and most of the measured values for patients with prostate cancer with advanced disease were within the reference intervals.

Association between plasma trefoil factors and clinical and pathologic data. There was no linear correlation between plasma trefoil factor values and Gleason sum. To further determine whether plasma TFF concentrations are correlated with the Gleason score, we stratified the patients with prostate cancer into two groups based on the Gleason score (≤6, n = 18 and ≥7, n = 56). A significant difference was determined (P = 0.02) in median TFF3 concentration indicating that higher Gleason grade tumors are associated with increased TFF3 concentrations.

Of the 42 patients with prostate cancer with hormone refractory or untreated advanced disease, 28 patients had verified bone metastases (Fig. 1). In these patients, plasma concentrations of TFF1, TFF2, and TFF3 were significantly increased (TFF1, P = 0.04; TFF2, P = 0.02; TFF3, P = 0.01) compared with the concentrations in plasma from the 14 patients with hormone refractory or untreated advanced prostate cancer without verified bone metastases.

Twenty-seven patients had localized disease and 42 patients had advanced prostate cancer. Using a cutoff of 200 pmol/L, the sensitivity and specificity of plasma TFF3 for detecting advanced disease was 74% (61-87%) and 81% (68-94%). Positive predictive value and negative predictive value were 82%
To test the diagnostic accuracy of plasma trefoil factors for identifying advanced prostate cancer, receiver operating characteristic (ROC) analyses were done (Fig. 2). The ROC curve of TFF3 resembles the curve of PSA, indicating an equal observed accuracy for TFF3 compared with PSA. ROC curves indicated a lower observed accuracy for TFF2 and also showed that the use of summed variables (TFF1 + TFF2 + TFF3, TFF1 + TFF2, and TFF1 + TFF3) did not improve the specificity and sensitivity of TFF measurements (data not shown).

Seven of the patients with clinically localized prostate cancer (20%) who underwent radical prostatectomy had preoperative TFF3 plasma concentrations >200 pmol/L (Fig. 1), but did not show signs of clinical or biochemical recurrence 1 year after surgery.

**Association between plasma trefoil factors and serum PSA.** PSA values exceeding 500 µg/L were routinely reported as “>500 µg/L” without further dilution of the samples. In patients with localized disease, and in patients with BPH where no PSA values exceeded 500 µg/L, no correlation was found between PSA and TFF1, TFF2, or TFF3 (Fig. 3). When the PSA results for all patient groups (n = 102) were pooled and the PSA values exceeding 500 µg/L were excluded (n = 10), a weak correlation between PSA and TFF3 was found (correlation coefficient = 0.33; P = 0.002). Additionally, after partitioning TFF3 values (n = 102) into two groups (PSA <10 µg/L, n = 34; and PSA ≥10 µg/L, n = 68) we could show that patients with PSA concentrations ≥10 µg/L had significantly increased plasma levels of TFF3 (P = 0.02).

Nine of 29 patients (31%) with advanced prostate cancer had low PSA values (four patients <4 µg/L and five patients <40 µg/L). In five of these patients (56%), TFF3 measurements exceeded 200 pmol/L.

**Immunohistochemistry.** In transurethral resection specimens from 10 patients with urinary flow obstruction due to prostatic hyperplasia, TFF1 immunoreactivity could not be detected. In the same specimens, TFF3 immunoreactivity was either absent or restricted to a few scattered dendriform cells in the basal compartment.

The patterns of immunoreactivity of TFF1 and TFF3 in radical prostatectomy specimens were similar to previous descriptions (5–7). In sections from 12 radical retropubic prostatectomy specimens, moderate to extensive expression of TFF1 and TFF3 was found in 10 and 12 cases. For TFF1, no difference in immunoreactivity could be observed between tumor tissue samples from primary radical retropubic prostatectomy cases (n = 12) and samples from invasive carcinomas. The invasive carcinomas from patients with advanced prostate cancer in five of eight cases, however, were only focally positive for TFF3 and the foci corresponded to neuroendocrine...
to the dimeric form of TFF3 (Fig. 4).

One sample from a patient with prostate cancer with localized disease showed the presence of TFF3 above the reported molecular mass of monomeric TFF3. In another sample, a major peak at 6.6 kDa corresponding to the TFF3 dimer (···) and a monomer (·) eluted at fractions 19 and 21, respectively.

**Discussion**

The discovery that trefoil peptides (TFF1 and TFF3) are overexpressed in prostate cancer tissue has suggested that these peptides may also be valuable for the screening of biological fluids. The novel and main findings in the present study of all three trefoil peptides in the plasma of patients with prostate cancer are that circulating TFF3 and TFF1, measured by the use of newly developed ELISA assays, are significantly increased in the plasma of patients with prostate cancer with advanced disease, and that increased plasma concentrations correlate with clinicopathologic variables. With the present data, there is clearly sufficient basis for more extensive studies to elucidate the potential clinical value of TFF3 as a marker of aggressive prostate cancer.

Recently, TFF3 was found to be consistently up-regulated in the majority of prostate cancer specimens as compared with benign prostate tissue in three data sets on mRNA profiling in prostate cancer (2–4, 28). Yet, in the subsequent immunohistochemical studies of TFF3 expression in prostate cancer tissues, no prognostic value of was found for TFF3 (5, 6). The plasma level of TFF3, however, may better reflect the actual tumor stage as it reflects the activity of not only the primary tumor but also its associated metastases.

At first sight, trefoil peptides do not exhibit the qualities of a potential useful prostate cancer plasma biomarker as they are expressed constitutively in mucous cells of the gastrointestinal tract, and it can be anticipated that the expression of trefoil peptides by these tissues may mask the protein expressed by the target tumor. Furthermore, prostate cancer may not be the sole contributor to increased circulating TFF as aberrant expression of trefoil peptides has been shown immunohistochemically in gastrointestinal inflammatory diseases and in other human cancers. We previously investigated serum TFF levels in patients with inflammatory bowel disease and inflammation and/or ulceration of the upper gastrointestinal tract (24, 25). Although we could show increased concentrations of serum TFF in these patients, most of the measured values (80% for TFF3) were within the reference interval. Our present studies show that the plasma levels of all three trefoil peptides were elevated in patients with prostate cancer with advanced disease. In all patient groups and in the healthy individuals, a significant positive correlation between the plasma concentrations of TFF1, TFF2, and TFF3 could be demonstrated (data not shown). For TFF1 and TFF2, large overlaps between the groups were present, which make them less valuable as clinical markers. Plasma concentrations of TFF3, however, fall within a consistent range in healthy males between the ages of 50 and 64 and in patients with BPH and were rarely elevated in these individuals in contrast to the patients with prostate cancer with advanced disease.

Gleason grade is one of the most powerful predictors of clinical outcome for patients with prostate cancer. We could not show a linear correlation of plasma TFF3 with Gleason grade. This lack of linear correlation might be due to small sample size. A major shift in prognosis occurs between Gleason scores of 6 and 7, and we could show that patients with Gleason scores ≥7 had significantly higher TFF3 concentrations than patients with lower Gleason scores. The data, however, also suggests that plasma TFF3 measurements may be an independent prognostic factor, which in combination with the Gleason score, may be used, e.g., in nomograms to predict pathologic stage.

Early dissemination of prostate cancer may occur despite local control of the disease. The finding that TFF3 was significantly increased in patients with prostate cancer with advanced disease and a positive predictive value of 81% (70-94%) raises the possibility that plasma measurements of TFF3, in combination with other pretreatment factors, might aid in determining who may or may not benefit from radical surgery. The observation that the immunohistochemical expression of TFF3 in specimens from patients with advanced prostate cancer corresponded with neuroendocrine differentiation, further indicates that this marker may have the ability to identify androgen-independent disease and that TFF3 may complement the PSA assay in the early detection of hormonal resistance in
patients with prostate cancer. Further studies will enable us to answer these questions and to determine if the TFF3 measured in plasma from prostate cancer patients arises from the prostate gland.

The physiologic functions of the trefoil peptides proven by numerous in vitro studies can be grouped under two broad headings: mucosal surface protection and repair after injury. The possible molecular mechanisms resulting from the increased TFF expression in cancer are unclear and it is not known whether trefoil factors are facilitating carcinogenesis by establishing a supportive tumor microenvironment or whether they simply represent passive markers of activated pathways. TFF peptides have been shown in vitro to enhance cell migration (mitogenic effect) and cell scattering, which both are effects that support the process of mucosal restitution but may be undesirable and deleterious during neoplastic progression.

In summary, the study presents data which supports the value of plasma determinations of TFF3 in the prediction of aggressive prostate cancer. Whether plasma TFF3 will prove itself as a marker that can be applied in clinical practice remains to be established in prospective studies.

Acknowledgments

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References

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