The influence of PSA-RNA yield on the analysis of expressed prostatic secretions (EPS) for prostate cancer diagnosis

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Introduction: *In patients with prostate cancer, luminal prostate-specific antigen (PSA) enters the circulation because the basement membrane and glandular epithelium are damaged. Given that excess mobilization of prostate cells during prostatic massage can influence normalization in diagnostic testing, we studied PSA mRNA levels in expressed prostatic secretions (EPS) from patients undergoing biopsy for prostate cancer to determine if prostate cells are preferentially mobilized from patients with prostate cancer during prostatic massage.*

Materials and methods: *Quantitative Reverse-Transcription PCR (qRT-PCR) was used to measure the RNA levels of GAPDH, PSA, TMPRSS2:ERG and PCA3 in EPS specimens obtained from patients undergoing biopsy for prostate cancer.*

Results: *The level of PSA mRNA is significantly elevated in EPS specimens obtained from patients with a subsequent diagnosis of prostate cancer. This correlation influenced diagnostic testing results from EPS in two ways. First, when used as an exclusion parameter it appears to improve the diagnostic performance of TMPRSS2:ERG in EPS. Second, when used as a normalization parameter it appears to decrease the performance of these same tests.*

Conclusion: *When comparing the results of mRNA based prostate cancer diagnostics in EPS it will be essential to consider PSA mRNA as a prostate specific gene and not a housekeeping gene.*

Key Words: RNA, prostate, diagnosis, human PCA3, human TMPRSS2-ERG fusion protein

Introduction

The introduction of widespread prostate-specific antigen (PSA) screening has dramatically improved the landscape of prostate cancer diagnosis and treatment. With the implementation of PSA screening, the Surveillance, Epidemiology, and End Results (SEER) database shows the incidence of metastatic prostate cancer at the time of diagnosis has dropped from 16% in the mid 1980's to 4% in 2003.[1] Even so, PSA screening for prostate cancer has recently been under scrutiny by the media, bureaucratic, and academic institutions because its limited sensitivity and specificity results in substantial overdiagnosis. For example, the usual indicators for prostate cancer (e.g., serum PSA > 4 µg/L and/or abnormal digital rectal examination [DRE]) require a prostate biopsy for definitive diagnosis. On average, approximately 62% of biopsies will initially be negative and 8% of these patients will undergo a repeat biopsy. The problem of unnecessary biopsies is further compounded by the potential risks of sepsis and hemorrhage associated with this procedure.[2] Recent data has also shown that 15% of men with the

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typical "normal range" of PSA (serum PSA < 4 µg/L) have prostate cancer, with a substantial incidence of high grade disease.[3] Therefore based on PSA screening, hundreds of thousands of men in the United States and other countries who do not have prostate cancer are nonetheless undergoing unnecessary biopsies because their PSA values exceed the threshold. Moreover, since there is no PSA value that ensures a patient does not have prostate cancer, many prostate cancers are not diagnosed because they are below the threshold.

For these reasons, there are many candidate biomarkers being studied to replace or supplement PSA and DRE for prostate cancer screening. The ideal biomarker should be disease specific, cost-effective, minimally invasive, and reproducible.[4] PCA3 (prostate cancer antigen 3) is the most widely studied of potential biomarkers for prostate cancer.[5-7] A recent study has successfully applied PCA3 to the prediction of prostate biopsy outcome in urine specimens obtained after attentive DRE.[5,6,8] Another well-characterized expression marker comprises the fusion TMPRSS2 to the ERG transcription factor. Type III TMPRSS2:ERG variants have demonstrated promising results as a post DRE urine biomarker in mixed populations of patients undergoing biopsy for cancer diagnosis or previously diagnosed patients undergoing surgery for prostate cancer.[9]

Previous work our laboratory has focused on expressed prostatic secretions (EPS) rather than post DRE urine as a source of potential biomarkers. We found EPS to provide a consistent source of adequate DNA and RNA for molecular marker detection by real time PCR assays.[10] Recently we compared the performance of Taqman QPCR assays designed to quantify prostate cancer biomarkers by reference to cloned standards. We found that a single EPS assay designed to detect both type III and type VI variants of TMPRSS2:ERG fusions was superior to PCA3 in diagnostic performance for the prediction of prostate biopsy outcomes.[11]

PSA mRNA in EPS specimens appears to arise from the prostatic stromal and epithelial content in prostatic secretions. In addition, the pathological constant in prostate cancer is damage to or absence of the epithelial basement membrane, which may facilitate cellular mobilization during massage. Because of the absence of the basement membrane, EPS collected from prostatic massage in patients with prostate cancer may contain increased levels of PSA RNA compared to those who do not have prostate cancer. For these reasons, we evaluated the influence of PSA yield on the performance of TMPRSS2:ERG as a marker for prostate cancer diagnosis.

Materials and methods

Eighty-six men who were undergoing prostate biopsy for evaluation of prostate cancer gave consent for EPS specimen collection under an Institutional Review Board (IRB)-approved, blinded, prospective study protocol. Before prostate biopsy, a DRE was performed with prostatic massage and milking of the urethra to collect prostatic secretions. The EPS was immediately placed on ice and then washed with cold 1X phosphate buffered saline and collected by centrifugation in three separate aliquots of equal volume. Pellets were stored at -80°C prior to analysis.

Reverse transcription Taqman QPCR

RNA was isolated from the pellets using the RNAqueous Kit (Ambion) according to the manufacturer's instructions. Total RNA was converted to cDNA as previously described.[10] Quantitative PCR was used to determine expression levels of type III and VI TMPRSS2:ERG fusions, PSA, GAPDH, and PCA3. Primers and reaction conditions have also been previously described.[10] In addition, plasmids containing cloned standards appropriate to each reaction were linearized by restriction digestion and serially diluted from stock solutions as described.[11,12] Standards were run in parallel in the same rotor as the unknowns. Results are expressed as the number of copies detected in a 25 µL reaction volume by comparison to the corresponding plasmid standard curve. Data points were assigned as zero if amplification was not observed during the 50 cycle PCR reaction.

Statistical analysis

Our objective was to determine the utility of EPS biomarker combination assays in discriminating between prostate cancer and benign prostate biopsies. We fit logistic regression models with benign versus prostate cancer as a response, and our explanatory variables were DRE, serum PSA level, PSA mRNA copy number, PCA3 mRNA copy number and TMPRSS2:ERG mRNA copy number. We also performed a sensitivity analysis in which we sequentially removed records with low PSA values from the logistic regression. We calculated receiver operating characteristic (ROC) curves and calculated the area under the curve (AUC) of the ROC curves. The ROC curves' AUCs were the metric for comparing the discrimination between a normal biopsy and prostate cancer using the assays. We report AUC values and asymptotic 95% confidence intervals.

TABLE 1. **Study population characteristics**

Parameter	Benign	Prostate cancer	Total
n	39	35	74
Mean age (SD)	64 (7.7)	67 (6.7)	65 (7.3)
Mean serum PSA (SD)	7.1 (5.0)	9.8 (15.9)	8.4 (11.6)
DRE, n (%)			
Normal	25 (64)	20 (57)	45 (60)
Not completed	4 (10)	4 (11)	8 (61)
Suspicious	10 (25)	11 (31)	21 (28)
Ethnicity, n (%)			
White	35 (90)	31 (89)	66 (89)
Other	4 (10)	4 (11)	8 (11)

Results

Biomarker data were collected in a blinded fashion from 86 patients. Our patient population has been described previously.[11] A brief description is provided in Table 1 and Table 2. Based on biopsy pathology, 35 patients were diagnosed with prostate cancer, 12 with high grade prostatic intraepithelial neoplasia (HGPIN) and 39 as benign. Patients with HGPIN but no evidence of prostate cancer were excluded (12 patients), which yielded a total of 74 patients.

RT-PSA measurements

In order to determine whether or not prostate cell mobilization by prostatic massage was correlated with the presence of prostate cancer we measured PSA mRNA levels in the blinded-patient cohort using RT-PCR. The results are depicted graphically in Figure 1. The average PSA yield on patients with a subsequent benign diagnosis was 6577 copies per 25 µL reaction, while patients with subsequent diagnosis of prostate cancer yielded an average of 8896 copies per reaction. When the copies were log-transformed and normalized to the log transform of the putative housekeeping gene

TABLE 2. **Subject characteristics**

Factor	n = 74
Age (median, range)	65 yrs (49-83)
Serum PSA (median, range)	5.9 (0.6-98.6)
PSA copy number (median, range)	820 (1-84800)
TMPRSS2:ERG (median, range)	37.7 (3.3-296)
Biopsy (% prostate cancer)	35 (47%)
Gleason score	
2 + 3 = 5	1 (3%)
3 + 2 = 5	1 (3%)
3 + 3 = 6	19 (54%)
3 + 4 = 7	9 (26%)
4 + 3 = 7	3 (9%)
4 + 5 = 9	2 (6%)
DRE (% suspicious)	21 (32%)

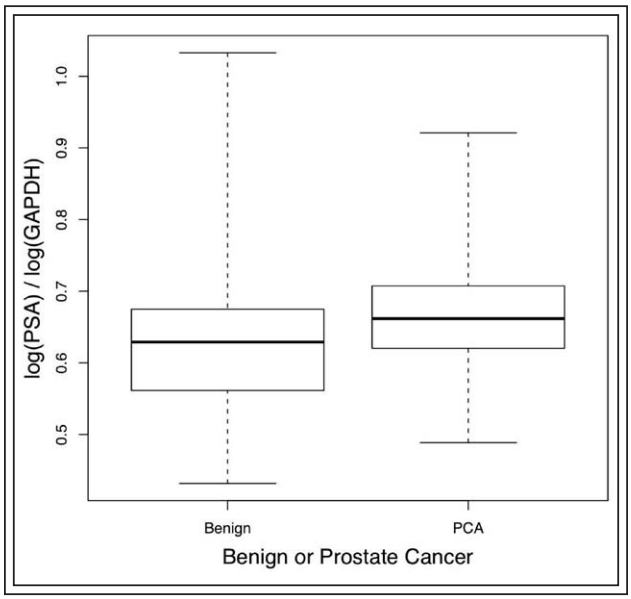

Figure 1. The ratio of PSA mRNA compared to the housekeeping gene GAPDH. Box and whisker plot of the log (PSA mRNA)/log (GAPDH mRNA) values for EPS specimens obtained from patients with a subsequent diagnosis of prostate cancer (PCA) compared with those for whom the subsequent diagnosis was did not detect prostate cancer (benign).

GAPDH, the mean PSA ratio was elevated by 5% in patients with prostate cancer, or 35% mean elevation in PSA yield with respect to copies per 25 µL reaction.

Logistic regression

The logistic regression (n = 74) on serum PSA and TMPRSS2:ERG gave an AUC value of 0.783 (95% CI: 0.689-0.877) from ROC analysis, Figure 2a. This is an improvement over the AUC value for serum PSA alone of 0.552 (95% CI: 0.439-0.665). However, when TMPRSS2:ERG was normalized to PSA mRNA, Figure 2a, its AUC value decreased from 0.783 (95% CI: 0.689-0.877) to 0.580 (95% CI: 0.468-0.692). Similar findings were obtained when logistic regression was carried out on serum PSA + PCA3 normalized to PSA mRNA, Figure 2b.

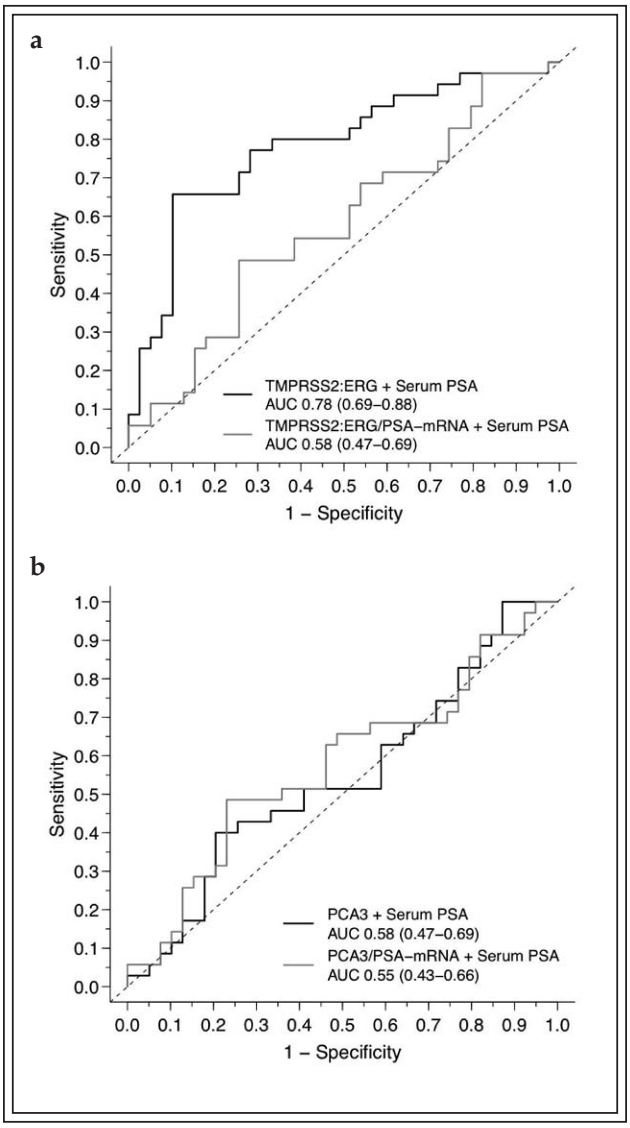

Figure 2. ROC curves comparing logistic regression analyses for serum PSA + raw or normalized biomarkers. **a)** TMPRSS2:ERG + Serum PSA; **b)** PCA3 + serum PSA.

We also performed ROC analyses to test the effects of exclusion of patient specimens with low PSA mRNA levels in EPS. The PSA mRNA copy numbers ranged from 1 copy to greater than 85,000 copies per 25 µL assay. Our analyses fit logistic models to the complete data set (n = 74) and to data sets that remove records with low PSA mRNA copy numbers. For example, if we include only patients with ≥ 50 copies of PSA mRNA per specimen (n = 61, 82% of subjects), the performance of serum PSA + TMPRSS2:ERG improves to an AUC of 0.790 (0.688-0.892). The performance of the assay continues to improve in a roughly linear fashion, Figure 3, as the exclusion PSA mRNA level is increased. For patients with ≥ 1000 copies of PSA mRNA in EPS (n = 35, 47% of subjects) the AUC for the logistic regression of serum PSA + TMPRSS2:ERG reached 0.914 (95% CI 0.821-1.000).

Discussion

The role of PSA expression in prostatic fluid samples is debatable in the analysis of new molecular markers. A current theme in the PCA3 literature suggests that PSA mRNA is the product of a reliable prostate specific "house keeping" gene[4] even though prostate specificity precludes a housekeeping function. In fact, normalization with PSA has been proposed since the first publication of the urinary PCA3 test.[13] However, our data suggest that PSA mRNA levels may indeed be an independent marker for prostate cancer due to damage to the basement membrane.

In prostate cancer, luminal PSA enters the circulation because the basal cell layer and the basement membrane of the glandular epithelium are damaged.[13,14] PSA mRNA found in EPS is expected to arise from luminal cells or cell fragments forced into the prostatic urethra during massage. Damage to the basal cell layer and basement membrane present in cancerous prostate glands is expected to facilitate cell mobilization during massage. Thus, EPS collected directly after prostatic massage or secondarily in urine collected after attentive DRE is expected to contain increased levels of PSA RNA when specimens are collected from patients with prostate cancer compared to those collected from normal patients.

Normalization of biomarker levels to PSA-RNA levels in EPS is expected to alter diagnostic results when employed in the manner generally described for PCA3 testing for two reasons. First, QPCR standard curves are generally determined based on the linearity of semilog

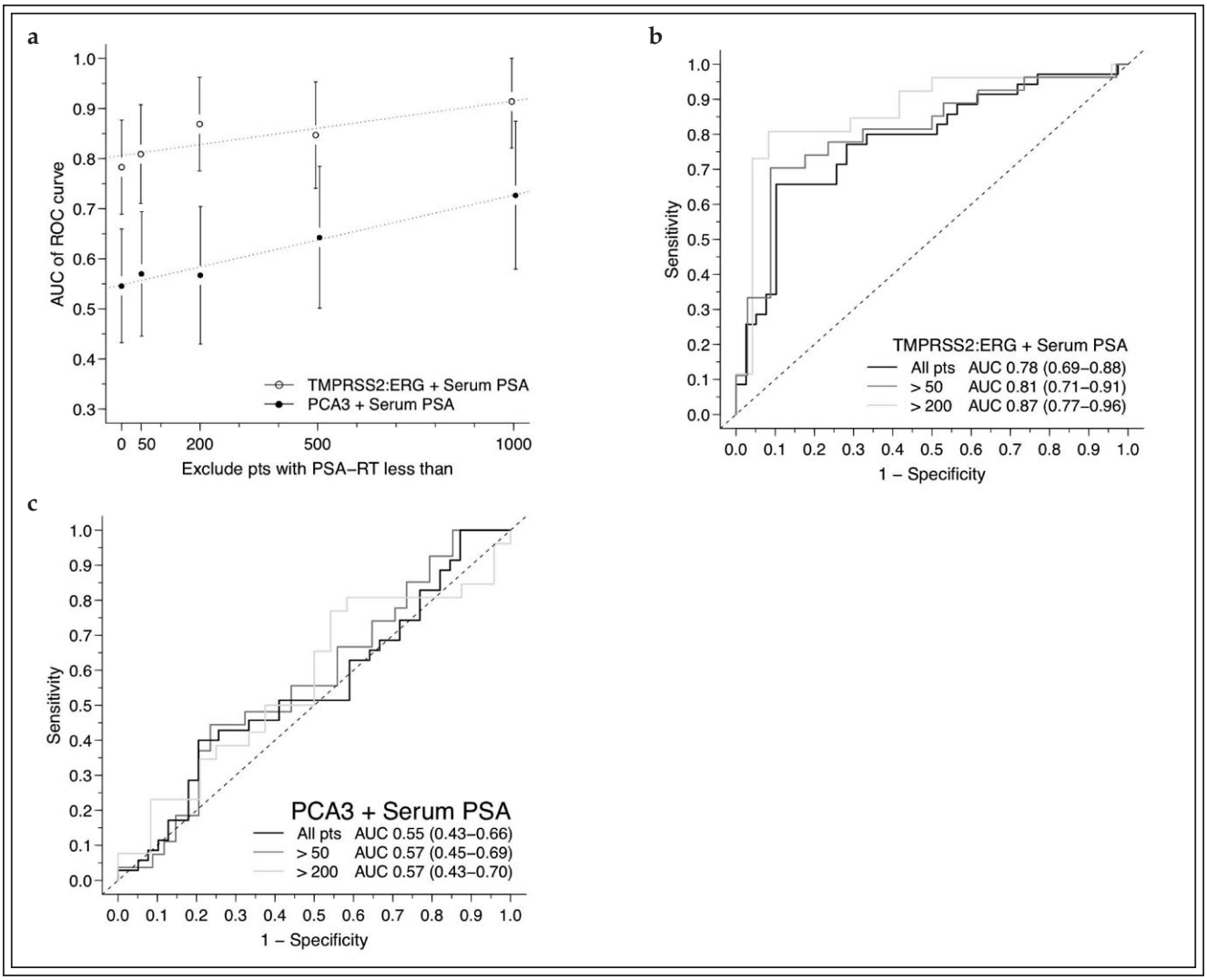

Figure 3. Effect of progressive exclusion of patients based on PSA-MRNA values. **a)** AUC values are plotted for logistic regression on TMPRSS2:ERG + serum PSA, or PCA3 + serum PSA for patient sets with more than the indicated value for PSA mRNA copy number in EPS; **b)** Graphical representation of the ROC curves for TMPRSS2:ERG + serum PSA exclusion levels up to 200 copies of PSA mRNA in EPS; **c)** Graphical representation of the ROC curves for PCA3 + serum PSA exclusion levels up to 200 copies of PSA mRNA in EPS.

data plots that fail to display the true uncertainty in individual determinations. This uncertainty can be significant and the uncertainty in the marker-RNA:PSA-RNA ratios will be compounded because the errors associated with each parameter will sum to give the error in the ratio.

Further, the excess mobilization of prostate cells that is expected from cancerous prostate glands was borne out by our data on RT-QPCR values for PSA mRNA from EPS specimens. Those values for the data set (n = 74) gave an average value of 6577 copies PSA mRNA per 25 µL reaction for specimens from patients with normal biopsies and 8896 copies from patients with positive biopsies. That is to say that for the EPS specimens analyzed here, the average content of PSA mRNA in patients who were later found to have a positive biopsy was higher than the average content of the PSA mRNA from patients who were later found to have a negative biopsy. Figure 1 shows the log ratio of PSA/GAPDH for EPS specimens from benign versus prostate cancer patients. The ratio for prostate cancer patients indicates a higher level of PSAmRNA (prostate specific) versus GAPDH (expressed in all cells). Indeed EPS specimens from prostate cancer patients appear to have a higher concentration of prostate cells compared to benign EPS specimens. Since prostate cancer cells

themselves produce about 1.5-fold less PSA mRNA than normal cells, EPS collection appears to mobilize more normal cells from tumor-bearing prostates. Thus any difference in a tumor marker that is elevated in the patients with positive biopsy would be suppressed by the normalization procedure recommended for PCA3 analysis in post massage urine.

Since our analyses thus far have been confined to EPS where a more vigorous massage is employed (about 30 seconds of attentive massage in our case) we cannot determine whether or not these effects are seen in post massage urine testing where exactly three strokes using firm pressure are applied to each lobe.[5] Moreover, it is difficult to determine from the literature whether this effect is seen with post massage urine specimens because the methods for determination of uninformative RNA levels are not immediately available from the literature.[4] However, the biology of prostate cancer and the nature of RT-QPCR results reported here argue against normalization of results to PSA mRNA determinations.

Conclusions

The stratification of PSA yield in our patient population clearly impacts the effectiveness of the TMPRSS2:ERG assay. Selecting for patients with more than 200 copies of PSA per specimen improved the AUC from a baseline value of 0.783 to 0.869. We believe that PSA stratification improves the positive predictive value for the TMPRSS2:ERG assay by selecting for a group who is more likely to have prostate cancer.

This study adds new insight to the potential role of PSA yield as a component of prostate marker assays. It also highlights the different characteristics between EPS and the post massage urine specimens described in the PCA3 publications. Clearly additional studies are needed to further determine the differences between EPS and post massage urine as a non-invasive source for biomarkers. □

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