Acta Oncologica, 2011; 50(Suppl 1): 85–89

informa
healthcare

REVIEW ARTICLE

Tumour markers in prostate cancer III: Biomarkers in urine

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Abstract

The serum PSA test still is the most important biomarker for the detection and follow-up of prostate cancer. PSA-based screening can reduce disease specific mortality but coinciding unnecessary testing and overdiagnosis warrant further research for more specific biomarkers. Numerous studies of both serum and urine-based prostate cancer biomarker candidates have been presented the last ten years. However, biomarkers for identifying the most aggressive subsets of this malignancy are still missing. Being non-invasive, urine-based tests might be suitable for both clinical and (mass) screening purposes, but also for prediction and to gain prognostic information. Protein-based, DNA-based and RNA-based urine biomarkers have been developed and tested. *Protein markers in urine*. Data on protein-based urine biomarkers (i.e. Annexin A3, matrix metalloproteinases and the urinary:serum PSA ratio) show up to now contradictory results and further studies are warranted to be able to assess their clinical value in which the cost aspect should not be overlooked. *DNA markers in urine*. Studies on DNA-based urine biomarkers focus on hypermethylation of gene panels with GSTP1 hypermethylation being the most promising individual marker. Larger prospective clinical studies of single markers and gene panels are however needed to validate their clinical utility. *RNA markers in urine*. RNA-based urine biomarkers are by far the most developed. The PCA3 test, the TMPRSS2–ERG fusion gene, transcript expression levels of *GOLPH2*, *SPINK1* and their combination have been subject of many studies showing encouraging results. *Conclusion*. Up to now urine-based biomarkers represent a promising alternative or addition to serum-based biomarkers. Prospective studies in a multivariate setting, including larger sample sizes and avoiding attribution bias caused by preselection on the basis of serum PSA are however required.

The serum PSA test still is the most important biomarker for the detection and follow-up of prostate cancer. The test is well tolerated, quick, cheap and standardised. Physicians are familiar with test results and can easily translate those into a certain risk level for having the disease or into the risk of tumour progression.

Recently it has been shown that screening with the use of the serum PSA test can reduce disease specific mortality [1–3]. This very important gain comes however with considerable costs such as 70–80% of potentially unnecessary prostate biopsies, depending on the PSA cut-off level used [1]. Next to this PSA-based screening leads to overdiagnosis; i.e. detection of "non-life threatening" disease, often resulting in overtreatment [4].

New biomarkers for the detection and staging of prostate cancer are therefore an absolute must, and numerous studies of both serum and urine-based prostate cancer biomarker candidates have been presented the last decade. However, biomarkers for identifying the most aggressive subsets of this malignancy are still missing. Being non-invasive, urine-based tests might be suitable for both clinical and (mass) screening purposes, but also for prediction and to gain prognostic information. Urine-based tests can roughly be divided into three groups; protein-based, DNA-based and RNA-based markers. This report will cover last years development in this area and highlight the most promising urinary markers in prostate cancer.

Protein markers in urine

Initially most research focused on the serum-urinary PSA ratio with conflicting results as compared to total serum PSA alone. In a prospective multicentre trial Irani et al. investigated the clinical relevance of the

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(Received 15 August 2010; accepted 6 September 2010)

ISSN 0284-186X print/ISSN 1651-226X online © 2011 Informa Healthcare
DOI: 10.3109/0284186X.2010.524935

urinary:serum PSA ratio in enhancing the specificity of serum PSA in the detection of prostate cancer [5]. In patients with a PSA level of 4–10 ng/ml, receiver operating characteristic curves (ROC) showed that the urinary:serum PSA ratio had a larger AUC (0.63) than did total PSA (0.55) or free-to-total PSA ratio (0.60). In a later prospective study, urinary:serum PSA ratio was significantly different, between patients with prostate cancer and those with benign prostatic hyperplasia, with ROC analysis showing a diagnostic cut-off for urinary PSA of $>$ 150 ng/ml, with a sensitivity of 92.5% [6]. Promising results from these two studies were contradicted by Pannek et al. who failed to show any improvement in prostate cancer detection or staging in a cohort of 110 men [7].

Annexin A3 (ANXA3) is a calcium-binding protein with decreased production in prostate cancer cells. Quantification by ANXA3 using Western blots of urine samples showed significantly lower values in prostate cancer patients as compared with BPH patients, resulting in an improved sensitivity at high specificities compared with total PSA [8]. The combination of PSA and urinary ANXA3 showed an AUC of 0.81 in the overall cohort.

Several studies have demonstrated a role of matrix metalloproteinases (MMPs) in growth, invasion and metastatic spread in prostate cancer and other malignancies [9]. In a recent study of 103 prostate cancer cases compared with 45 healthy controls, Roy et al. showed that MMP9 in urine was an independent predictor of prostate cancer on multivariate analysis and that prostate cancer could be detected with a specificity of 82% and a sensitivity of 74% with regard to the presence of any MMP in urine [10].

As concluded by Ploussard and de la Taille [11], more recently described urinary protein markers like delta-catenin [12], the heptatocyte growth factor (c-met) [13] and thymosin β15 [14] have been evaluated in different pilot studies but none of these proposed new prostate cancer markers have yet been validated in independent studies.

The availability of proteomic platforms allowing the analysis of hundreds of peptides simultaneously protein-based urinary marker research has evolved enormously during the last decade. However the complexity of peptide analysis in urine samples was illustrated by Adachi et al. [15] who demonstrated the occurrence of more than 1 500 proteins in the normal urine proteome. A comprehensive review on proteomics and prostate cancer was recently published by Goo and Goodlet [16] and this area of research will not be further described in this report.

Metabolomics is a new approach to identify and separate metabolites using technology similar to proteomics. These small molecules are often the final products of biochemical activity in molecular pathways and metabolomics could potentially provide opportunity to develop diagnostic and prognostic evaluation to stratify patients to choose the best kind of treatment. Using a combination of high-throughput liquid and gas chromatography-based mass spectrometry, Sreekumar and colleagues identified 1 126 metabolites across 262 clinical samples (plasma, tissue and urine) and proposed sarcosine, an *N*-methyl derivative of the amino acid glycine, to be an important biomarker for prostate cancer progression and metastasis [17]. Other groups, using somewhat different methodology, have failed to reproduce these findings [18] and the value of sarcosine as an important biomarker in prostate cancer is still under debate.

Taken together, contradictory results are reported on protein-based urinary markers and further studies are warranted to be able to assess their clinical value in which the cost aspect should not be overlooked.

DNA markers in urine

Hypermethylation at various gene loci has been associated with most malignancies, and several DNA methylation markers have been investigated in prostate cancer. The loss of glutathione-*S*-transferase P (*GSTP1*) expression as a result of promoter hypermethylation is the most common molecular alteration reported in prostate cancer [19]. Initial studies reported promising results with high sensitive and moderate specificity in assays detecting GSTP1 hypermethylation in urine samples, whereas subsequent studies have shown conflicting results in terms of predictive accuracy as recently reviewed by Ploussard and de la Taille [11]. Later reports have mainly focused on hypermethylation of gene panels again showing contradictory results [20–22]. A multiplexed, quantitative methylation-specific PCR assay consisting of the three different methylation markers, GSTP1, RARB and APC was recently tested in a prospective multicentre study of post digital rectal examination (DRE) urine samples from 178 patients with prostate cancer and 159 controls [23]. The predictive accuracy (area under the curve, AUC) of the assay for detecting prostate cancer was 0.72, but this was only a marginal gain in predictive capability with respect to biopsy outcome as compared to total PSA and DRE alone. Results this far have suggested that gene methylation might serve as a useful marker in prostate cancer, and that GSTP1 hypermethylation is the most promising individual marker. Larger prospective clinical studies of single markers and gene panels are needed to validate their clinical utility.

RNA markers in urine

RNA-based urinary tests are by far the most developed. The well known differential display clone 3 or

PCA3 test is already commercially available under the trade name Progensa® PCA3 (Gen-Probe, San Diego, Ca) [24].

The PCA3 test has been subject of many studies virtually all showing superiority of the PCA3 score to the serum PSA level in predicting biopsy outcome when comparing ROC curves. Especially the high specificities in the range of 80 to 90% are impressive and can be helpful in avoiding unnecessary biopsies. Looking into more detail at test performance characteristics and the study cohort used is however warranted. Sensitivity and specificity of the PCA3 test in men with PSA levels in the so-called grey zone (4–10 ng/ml), representing those men that actually would benefit from an additional test are not that convincing. In a comprehensive review of Vlaeminck-Guillem et al. the test performance in the PSA grey zone is summarised [25]. The specificity when applying different, study dependent, PCA3 cut-off values is indeed impressive ranging from 71 to 93%. However sensitivities are in the range of 53 to 84% [24,26–28] and question its clinical value. Data for men with previous negative biopsies on the basis of an elevated PSA level and/or abnormal DRE result show a similar picture. Again specificities are high and repeating of unnecessary biopsies will without doubt be avoided with the use of the PCA3 test. However with corresponding sensitivities ranging from 47 to 75% one can question whether this is desirable, especially in these men [26–30].

Even more important to realise when interpreting these data is the fact that studies of new diagnostic markers are subject to attribution or assignment bias. Usually a more or less arbitrarily chosen cut-off value is used as a "gold standard" to determine the indication for the decisive test, a prostatic biopsy, and the assumption is made that no cancers are present below that cut-off value. This assumption has been proved wrong by findings in the control arm of the Prostate Cancer Prevention Trial (PCPT), where more than 5 000 men were biopsied independent of their PSA status. As an example: a PSA cut-off value of 4.0 ng/ml, a commonly used biopsy threshold, missed about 75% of all biopsy-detectable prostate cancer [31]. Most reported studies on PCA3 are in men previously screened with PSA and selected on the basis of an elevated PSA level. When calculating sensitivity and specificity all biopsy detectable prostate cancer cases present below the PSA cut-off are thus ignored. Resulting sensitivity and specificity percentages are actually relative sensitivity and specificity values.

Recently a side study within the ERSPC Rotterdam evaluated the value of PCA3 as a first line screening test. The study design was chosen as such that more than 80% of men had a biopsy indication. Prostate biopsy was indicated if the PSA level was $\geq$ 3.0 ng/ml and/or the PCA3 score was $\geq$ 10. With doing so the attribution bias was avoided as much as reasonably possible [32]. Based on ROC analyses of 721 men all biopsied, PCA3 performed marginally better than total PSA in predicting biopsy outcome. AUCs of PSA and PCA3 were 0.58 and 0.64 ($p=0.143$) respectively. A sensitivity of 85% coincided with a PSA cut-off level of 1.0 ng/ml and a PCA3 score cut-off of 20. Specificities were similar and relatively low; 27% and 28% respectively. Again high specificities coincided with low sensitivities in both PSA and PCA3. For example a specificity of 75–80%, reached with a PSA cut-off of 4.0 ng/ml or a PCA3 score cut-off of 60 rendered sensitivities of 24% and 39% respectively. These data indicate that the PCA3 test, although to a somewhat lesser extent suffers from similar weaknesses as the serum PSA test. There is no cut-off value at which sensitivity and specificity achieve a reasonable balance.

The relationship between the PCA3 score and parameters of cancer aggressiveness has also been studied and differ by outcome. Some studies [33] report a positive relationship between PCA3 scores and parameters of more serious disease, while other studies could not find such a relationship [34].

Another RNA-based urinary biomarker comes from gene fusions which occur in approximately 50% of the prostate cancer cases. The most common fusion in prostate cancer is between the strong androgen-regulated TMPRSS2 gene transcriptional promoter and the oncogene ERG. This fusion results in an androgen-regulated TMPRSS2–ERG fusion gene. In 108 men with prostate cancer biopsied on the basis of an elevated PSA level ($\geq$ 3.0 ng/ml) Hessels et al. analysed the fusion transcripts in urinary sediments. Again sensitivity was low (37%) with a very high specificity of 93%. No significant relationship was found between the presence of the fusion transcripts and Gleason score in prostate biopsies [35]. This was confirmed by Rice et al. who detected ERG mRNA in urine samples from 237 men [36].

Combining the PCA3 test and the TMPRSS2-ERG fusion test seems a way to improve diagnostic accuracy. A study done in 105 men showed that the PCA3 alone had an AUC of 0.65, while the combination of PCA3 and TMPRSS2-ERG increased the AUC to 0.77. Adding the PSA level to this multivariate approach resulted in an AUC of 0.80 [37].

If a multiplex analysis might improve the sensitivity of urine-based tests, without sacrificing specificity was evaluated by Laxman et al. [38] who performed quantitative PCR after whole transcriptome amplification in urine samples from 257 patients, including 152 men with prostate cancer and 105 with negative biopsy results. Different mRNA biomarkers were evaluated based on bioinformatic analysis and in univariate

and multivariate analyses, transcript expression levels of *GOLPH2*, *SPINK1*, *PCA3*, and *TMPRSS2–ETS* fusion significant predictors of prostate cancer, with an AUC significantly greater than that of the *PCA3* score alone (0.758 versus 0.662). *SPINK1* expression in urine samples was higher in *TMPRSS2–ERG*-negative than in *TMPRSS2–ERG*-positive samples, suggesting the mutual exclusivity of *SPINK1* expression and *ETS* fusions similar to tissue biomarker studies [39]. Validation studies are to be presented.

Conclusion

Urinary biomarkers for prostate cancer are subject of ongoing research and represent a promising alternative or addition to serum-based biomarkers. Prospective studies including larger sample sizes and avoiding attribution bias caused by preselection on the basis of serum PSA are however required. Looking at test performances combinations of different, both serum and urinary-based markers in a multiplex setting will most likely help in resolving the current problems in the (early) detection and staging of prostate cancer.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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