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14. ABSTRACT

Hypothesis: The overall objective is to provide, for routine clinical use, a simple and reliable method for identifying patients with prostate cancer so that transrectal ultrasound guided biopsies can be restricted to men harboring PCa. Our broad strategy will involve the use of selected markers for sensitive detection of cancer cells in ejaculate. **Objectives:** (1) To recruit patients for a prospective clinical trial in a screening assay of patient ejaculate and urine samples from which PCa cells will be isolated by (2) immunocapture using antibodies to specific cell-surface markers and (3) detected by real-time PCR, with a combination of genes upregulated in PCa. These methods will be refined for application for routine laboratory use. Logistic regression analysis will be used with the above combination of PSMA, DD3 and Hepsin to determine each patient's probability of harboring PCa. This data will be correlated with clinical data obtained by conventional diagnostic methods. **Relevance:** It is our strong expectation that this research will lead to provision of a simple and reliable approach for diagnosing PCa, in the longer term reducing the need for the invasive, imprecise and unpleasant procedure of TRUS-guided prostatic biopsies. In addition we expect that the earlier detection will be associated with and even greater proportion of cancer localised to the prostate at time of diagnosis.

15. SUBJECT TERMS
Detection of Prostate Cancer in its earliest stages of development, Non-invasive, Clinical Trial, Molecular Biomarkers

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Table of Contents

ANNUAL REPORT

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DEVELOPMENT OF A NOVEL, NON-INVASIVE DIAGNOSTIC TEST FOR PROSTATE CANCER

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KEY WORDS: DETECTION OF PROSTATE CANCER IN ITS EARLIEST STAGES OF DEVELOPMENT. NON-INVASIVE, CLINICAL TRIAL, MOLECULAR BIOMARKERS.

INTRODUCTION

Technical Abstract

Background: Prostate cancer (PCa) is the most common cancer diagnosed in men and is the second commonest cause of male cancer deaths throughout the western world. The oft quoted line that 'more men die with PCa than from it' misleads, falsely implying an innocuous nature. PCa is only curable when localized, which has been aided by detection of this disease earlier as a consequence of introduction of the prostate specific antigen (PSA) blood test. However, PSA is not a test for PCa even though the large majority of patients present with elevated serum PSA levels, in the absence of any other discernable abnormality. TRUS-guided prostate biopsies provide the second part of the contemporary two-step diagnostic process, which has proven to be a methodical approach to obtaining tissue cores for standard histopathology. However, TRUS-guided biopsies remain invasive, unpleasant and imprecise so that different strategies are warranted if a quantum leap in early detection is to be made. Our innovative approach has been to focus on ejaculate and urethral washings (voided urine immediately following ejaculation) from PCa patients as means of sampling abnormal prostate cells.

Objective/Hypothesis: The overall objective is to provide, for routine clinical use, a simple and reliable method for identifying patients with prostate cancer (**PCa**) so that transrectal ultrasound (**TRUS**) guided biopsies can be restricted to men harboring PCa. Our broad strategy will involve the use of selected markers for sensitive detection of cancer cells in ejaculate.

Specific Objectives: (1) To recruit patients for a prospective clinical trial in a screening assay of patient ejaculate and urine samples from which PCa cells will be isolated by (2) immunocapture using antibodies to specific cell-surface markers and (3) detected by real-time PCR, with a combination of genes upregulated in PCa.

Study Design: We plan to study specimens from 100 men whose TRUS biopsies reveal PCa and from 100 men whose ≥8 biopsies do not. At least 40 of the PCa patients selected will undergo retropubic radical prostatectomies, which will permit closer correlation with histological parameters and markers of progression. Both ejaculate and post-ejaculate voided urine (urethral washings) samples will be collected prior to TRUS biopsies or at least 2 weeks after this procedure. We will continue with our current strategy of immunocapture of PCa cells by targeting PSMA and other highly-expressed PCa cell-surface antigens. cDNA will be synthesised from the captured cells and Quantitative Real-time PCR will be performed with β2-microglobulin (housekeeping gene), PSMA, DD3 and Hepsin primer pairs. These methods will be refined for application for routine laboratory use. Logistic regression analysis will be used with the above combination of PSMA, DD3 and Hepsin to determine each patient's probability of harboring PCa. This data will be correlated with clinical data obtained by conventional diagnostic methods.

Relevance: It is our strong expectation that this research will lead to provision of a simple and reliable approach for diagnosing PCa, in the longer term reducing the need for the invasive, imprecise and unpleasant procedure of TRUS-guided prostatic biopsies. In addition we expect that the earlier detection will be associated with an even greater proportion of cancer localised to the prostate at time of diagnosis.

TIMELINE

Process	Year 1	Year 2	Year 3
Aim 1. - Patient recruitment	→	→	→
Aim 2. - Manufacture of polyclonal antibodies.	→		
- Optimisation of immunocapture protocols	→	→	
Aim 3. - Screening of patient samples with selected biomarkers		→	→
- Analysis of data and correlation with clinical outcomes.			→

RESEARCH BACKGROUND

Prostate cancer (**PCa**) is the most common cancer diagnosed in men and is the second commonest cause of male cancer deaths throughout the western world. However, its significance as a major health issue is underestimated. **The oft quoted line that 'more men die with PCa than from it' misleads, falsely implying an innocuous nature.** The morbidity that PCa exacts from middle-aged and elderly men, both from the disease itself and its various treatments is considerable, the magnitude of unwanted effects (and costs) escalating with advancing stage of disease[1].

PSA

PCa is only curable when localized. Detection of this disease earlier as a consequence of introduction of the prostate specific antigen (**PSA**) blood test, acknowledged by the NCI as a factor contributing to lowering the mortality rate over the past few years[1,2], has provided a diagnostic lead-time estimated by Gann *et al*[3] to be 5.5 years. In the mid to late 1980s only one third of PCas were diagnosed at curable stages compared with today when 80% are organ confined-and curable [4-6].

However, PSA is not a test for PCa. The large majority of patients detected present with elevated serum PSA levels, mostly between 4-10 ng/ml[7], in the absence of any other discernable abnormality. Although the prevalence of PCa depends on the population examined, most men undergoing prostatic biopsies have negative histology for cancer, even when ≥8 transrectal ultrasound (TRUS)-guided biopsies are taken[8-10], with biopsies repeated if suspicion of an undetected malignancy is high[7].

Despite employing PSA velocity, free/total PSA ratios, transition zone and total prostate volume/PSA relationships to try to identify those patients harbouring PCa, the diagnostic predictability of PSA remains disappointingly deficient when applied to individual patients[11-14]. **Furthermore with earlier detection of PCa through PSA testing, even when the tumour is thought to be localised, up to 25% of men have non-localised disease[15] prompting suggestions of a PSA cut-off of ≤3 ng/ml.** However apart from a reducing specificity, with PSA values between 3 and 4 ng/ml, approximately 15% of PCas detected will have extracapsular growth[16]. **Consequently the need for a simple test for PCa, which PSA in all its forms and guises is not, is warranted more than ever.**

TRUS

The second part of the contemporary two-step diagnostic approach is TRUS-guided prostate biopsies, with ultrasound imaging permitting spatial positioning of spring-loaded biopsy needles to provide a methodical approach to obtaining tissue cores for standard histopathology. **With few exceptions, TRUS imaging by itself is non-diagnostic.** The number of biopsy cores taken is important with the chance of missing a cancer by standard sextant biopsy estimated at about 25%[17] such that, more recently, the numbers of cores recommended are at least 8 and preferably a minimum of 10[1,18,19].

The issue of repeat biopsies was addressed by Djavan *et al*[19], particularly in relation to when it is reasonable to stop. Cancer detection rates in 1051 men biopsied were 22%, 10%, 5% and 4% with 1-4 TRUS biopsy sessions with 58%, 60.9%, 86.3% and 100%, respectively, having organ-confined disease. However, if further concerns regarding the possibility of undetected PCa arise after an interval of time, the utility of further TRUS-biopsy procedures is limited in terms of patient acceptance. Although the dreaded complication of life-threatening sepsis is fortunately uncommon, **TRUS-guided biopsies remain invasive, unpleasant and imprecise so that a different approach is warranted if a quantum leap in early detection is to be made.**

Molecular Biomarkers

Although the introduction of PSA and TRUS biopsies have resulted in prostate cancer being diagnosed much earlier and at a lower stage than ever before, a point of limiting returns appears to have been reached with respect the probability of further significant advances in lead-time detection of organ confined disease being achieved. One approach to overcoming these particular hurdles has been to identify other PCa specific biomarkers, which can be used to enhance the contemporary two-step diagnostic approach, thereby resolving some of the ambiguity inherent in both PSA testing and TRUS biopsies, particularly relevant for the 25% of patients who harbor occult metastases at time of diagnosis.

Protein Biomarkers

In addition to PSA, a number of protein markers have been reported. Prostate specific membrane antigen (PSMA), also known as glutamate carboxypeptidase II is an enzyme, which digests polyglutamyl folates to monoglutamyl folates[20]. PSMA was initially cloned from an androgen-dependent prostate cancer cell line, has since been detected in both benign and PCa tissues and in clinical terms is generally regarded as prostate-specific[21]. Further research with PSMA has focussed on using this antigen as a target for both Dendritic Cell based immunotherapies[22-24] and radioimmunotherapy using radiolabelled monoclonal anti-PSMA antibodies[25,26].

Hepsin, a transmembrane serine protease which, although widely expressed throughout the body, is up-regulated in malignant prostatic epithelium. Hepsin was identified by gene expression analysis of a large cohort of PCa tumours using microarray technology by 3 independent groups[26-30]. This gene displayed significantly increased expression in malignant tissues compared with normal and BPH tissues with highest expression in organ-confined, lower Gleason grade disease[30], making it a good candidate for early detection.

Genetic Biomarkers

The expression of both PSMA and Hepsin has also been demonstrated to be up-regulated by cDNA profiling of tumors[31,32] compared with BPH. This approach has identified many genes whose expression patterns are altered in malignant tissues. One such gene is DD3, which is a non-coding mRNA transcript, with one specific splice variant highly over-expressed in PCas[33]. Northern blot analysis showed that DD3 is expressed at a low level in normal prostate but is highly expressed in tumour tissues[33]. Subsequent RT-PCR quantification of DD3 confirmed its expression in the prostate but not in 21 selected other tissues or cell types (including leukocytes)[33]. Moreover, the diagnostic and prognostic value of DD3 has been determined in 2 separate studies, utilising tumour tissue and prostatic washings[34,35]. RT-PCR detection of DD3 in prostatic washings was shown to be highly specific for PCa (93%), with 67% sensitivity[36].

The focus of recent studies has changed from identification of individual biomarkers to utilising combinations of known PCa-specific markers, such as those described above, as predictors of disease recurrence following treatment with curative intent[37,38]. Our strategy has been to apply a number of selected biomarkers for PCR based detection of PCa cells in a non-invasive diagnostic screening assay for PCa.

KEY RESEARCH ACCOMPLISHMENTS

The overall objective of this project is to provide, for routine clinical use, a simple and reliable method for identifying patients with prostate cancer, so that transrectal ultrasound guided biopsies can be restricted to men harbouring prostate cancer (**PCa**). Our broad strategy will involve the use of selected markers for sensitive detection of cancer cells in ejaculate.

The specific objectives and corresponding tasks are:

Aim 1/Task 1. To continue to recruit patients for a prospective trial

Aim 2/Task 2. To uitilise immunocapture of PCa cells using antibodies to specific cell-surface markers

Aim 3/Task 3. To employ real-time PCR with a combination of genes upregulated in PCa in a screening assay of patient ejaculate and urine samples.

Task 4. To provide a simple and reliable approach for diagnosing PCa through the combination of the above methodologies

There have been a number changes to our research strategy since receiving the grant. The first has been to collect prostatic fluid obtained in urine following Digital Rectal Examination (DRE) in ***addition*** *to prostatic fluid obtained from ejaculate and urine immediately following ejaculation (See Task 1 below) . This decision was prompted by the published results of the multi-centre study by Fradet et al*[39] *and the commercialisation of test for PCA3/DD3 RNA in prostatic cells in urine immediately following DRE, by Bostwick Laboratories (http://www.bostwicklaboratories.com/;*[36,40]*).*

We believe that cells shed during DRE can only sample those areas of the prostate massaged by DRE. However, the vigorous muscular contraction of the prostate during ejaculation permits shedding of PCa cells from throughout the prostate gland. Therefore, we hypothesise that PCa cells, evident in ejaculate and post-ejaculate urines, will provide a more comprehensive sampling of the prostate than post-DRE urine alone, and therefore we wish to compare these two methods for sampling PCa cells. In addition, detection of DD3/PCA3 positive cells in post-DRE urines will act as our gold standard for optimising the methodology in ejaculate and post-ejaculate urines.

The Second change has been to utilise NASBA (Nuclic-Acid Sequence Based Amplification[41]*) to detect prostate cells secreted in ejaculate and urethral washings. Fradet et al* [39] *utilised this technique for detecting PCA3/DD3 in post-DRE urines, with a high degree of sensitivity.*

We are using the NASBA approach for PCA3/DD3 and PSMA, Hepsin and Gal-NAc-T3 in all 3 post DRE urine, ejaculate and post-ejaculation urines. In addition to using the post-DRE urine results for PCA3/DD3 as a reference for this and the other markers in combination in all 3 specimens, there is the added advantage of greater sensitivity with the NASBA approach in comparison with that provided by antibodies, or RT-PCR as outlined in our application.

Task 1. **To continue to recruit patients for a prospective clinical trial. (Months 1 – 36)**

a. We will recruit 100 men whose TRUS biopsies reveal PCa and from 100 men whose ≥8 biopsies do not. At least 40 of the PCa patients selected will undergo retropubic

radical prostatectomy, which will permit closer correlation with histological parameters and markers of progression. (Months 1 – 30)

b. Ejaculate and urethral washings will be obtained prior to TRUS biopsies or at least 2 weeks after this procedure. (Months 1 – 30)

c. Clinical data will be collected from each patient such that relevant comparisons can be drawn between clinical outcomes and gene expression levels. (Months 1 – 36)

PROGRESS. To date (months 1- 12) we have recruited 44 men who have provided Ejaculate with Post-ejacualate Urines and/or Post-DRE urines (see above). Clinical Data has been collected and is routinely updated for all of these individuals. In total we have obtained 30 post-DRE urines, 44 ejaculate samples and 44 post-ejaculate urine specimens: 3 patients have undergone radical prostatectomy for correlation with our TRUS biopsy and radical prostatectomy histology reference data.

As a result of the close collaborative spirit in out local prostate research committee, we have secured the support of the two largest private pathology companies in South East Queensland whose couriers promptly complement our local in-house staff for delivering specimens for analysis. We have also established collaborations with Clinical Urologists who will be referring patients for our study in the New Year, doubling our referral centres.

Task 2. To utilise immunocapture of PCa cells using antibodies to specific cell-surface markers (Months 1 – 36).

As stated above in the introduction, we are using the NASBA technique in the form of the Nuclisens-EasyQ™ Basic kit (Biomerieaux Australia, Sydney, Australia) for detection of prostate cells in ejaculate, post-ejaculate urine and post-DRE urine. Publications from the end of 2004[39,40]*, prompted us to reassess our methods for isolating prostate cancer cells. These studies found that if the molecular detection method was sufficiently sensitive, PCa cells could be detected in whole cell populations from post-DRE urines.*

Our own preliminary work, midway through 2004, found that conventional RT-PCR following total RNA amplification detected Hepsin, PSMA, and PSA transcripts in ejaculate and post-ejaculate urines following immunocapture with PSMA specific antibodies. However, this process was not sufficiently sensitive for detecting DD3/PCA3 in the same samples. Therefore, rather than pursue the immunocapture protocols detailed in this task, we have focussed on improving our detection technique by applying the NASBA methodology to our selected biomarkers, DD3/PCA3, PSMA, Hepsin and PSA[42]*.*

This methodology has the advantage of being performed on total RNA, without additional steps of reverse-transcription and the amplification products are detection in real-time using fluorescent molecular beacons[39]*. This process is quick and efficient as well as highly sensitive.*

Therefore the modified aims of this task are:

a. Design of molecular beacons for Prostate-cancer specific biomarkers, DD3/PCA3, PSMA and Hepsin, and for the prostate-specific marker KLK3 (PSA) according to the Nuclisens Basic kit User Manual © Biomerieaux, 2003 (Months 1 – 3)

b. Optimisation of NASBA-based fluorescent detection of cancer-specific transcripts (above) from cancer samples (Months 3 – 9)

c. Detection of the above biomarkers on ejaculate, post-ejaculation urine and post-DRE urine samples (urethral washings) as above. (Months 9 - 36)

d. Multiplexing the NASBA-based fluorescent detection for DD3/PCA3, PSMA, Hepsin, and KLK3 (PSA) in a single reaction for routine use in the laboratory. (Months 12-16)

PROGRESS: To date (months 1-12), we have the amplification and detection optimised for DD3/PCA3 (FAM-6-Molecular Beacon) and PSA (JOE-Molecular Beacon) –see Figure 1 below. We are currently refining detection of Hepsin (Rox-Molecular Beacon) and PSMA (Cy5-Molecular Beacon).

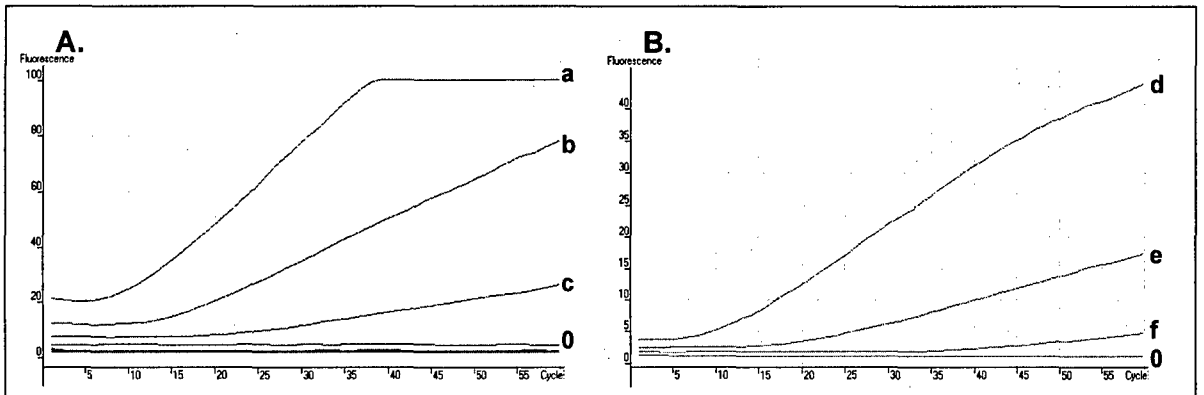

Figure 1. Standard Curves with serial dilutions of target RNA for **A.** DD3/PCA3, **a.** 1 pg/µl, **b.** 0.1 pg/µl, **c.** 0.01 pg/µl, **0.** No template control; and **B.** PSA **d.** 10 pg/µl, **e.** 1 pg/µl **f.** 0.1 pg/µl **0.** No template control.

In addition, we have preliminary data for detection of DD3/PCA3 in ejaculate and post-ejaculate urines using the above technique. 40 samples (from 20 individuals) were assessed and DD3/PCA3 positive transcripts were detected in samples from 7 individuals. An example of the results is shown in Figure 2. The same samples will be assessed for PSA, and the data will be collated with available clinical information for these patients.

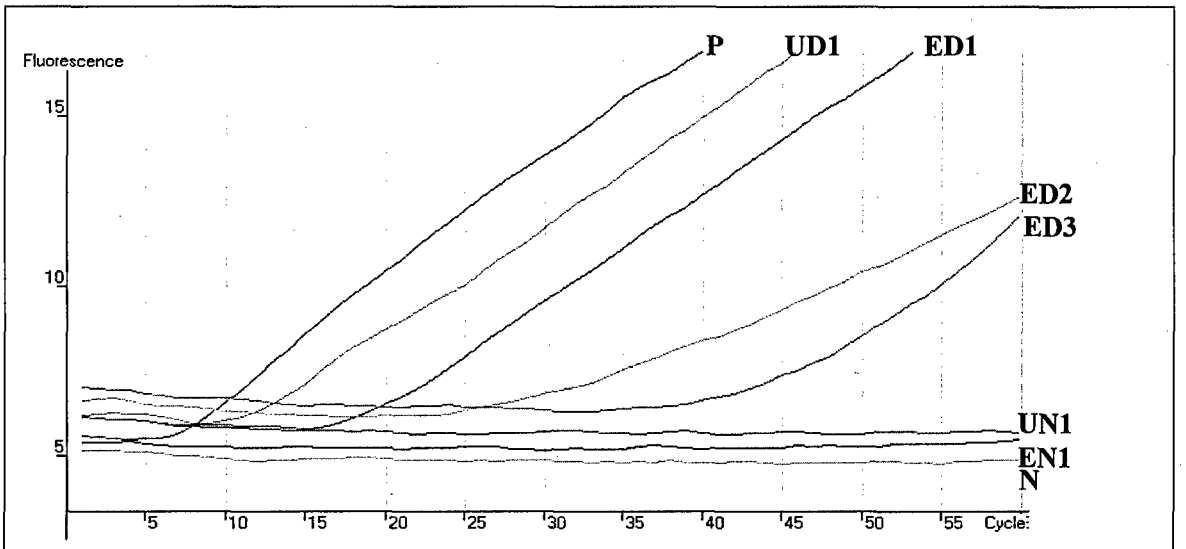

Figure 2: P; **Positive Control RNA,** N, **Water only negative control:** ED1, ED2, ED3, **DD3 positive Ejaculate RNA;** UD1, **Post-ejaculate-Urine DD3 positive RNA:** EN1, UN1, **DD3 negative Ejaculate and Urine RNA (respectively).**

Task 3. **To employ real-time NASBA, with a combination of genes upregulated in PCa, in a screening assay of patient ejaculate and urine samples (Months 12 – 36).**

a. We will isolate mRNA in PCa cells enriched from ejaculate and post-ejaculate urine samples collected from 100 PCa patients and 100 PCa negative donors. Real-time NASBA will be performed with PSA (prostate-specific gene), PSMA, DD3/PCA3 and Hepsin (PCa specific biomarkers). (Months 12 – 30)

b. As above, we will isolate mRNA in PCa cells enriched from post-DRE urine samples collected from the same 100 PCa patients and 100 PCa negative donors Real-time NASBA will be performed with PSA (prostate-specific gene), PSMA, DD3/PCA3 and Hepsin (PCa specific biomarkers). (Months 12 – 30)

c. Mean expression levels in genetic markers will be compared between PCa-affected and unaffected patients using t-tests and/or Wilcoxin non-parametric tests. Logistic regression analysis will be conducted to assess the usefulness of gene expression as a screening tool. (Months 30 – 36)

PROGRESS: As stated above, we have preliminary data for detection of DD3/PCA3 in ejaculate and post-ejaculate urines using the real-time NASBA technique. This data will be stored for analysis with additional data collected from samples from a total of 200 patients.

Task 4. **To provide a simple and reliable approach for diagnosing PCa through the combination of the above methodologies. (Months 12 – 36)**

a. Sensitive detection and quantitation of PCa cells with a combination of prostate cancer specific genetic biomarkers. (Months 12 – 30)

b. Using statistical methods to draw relevant comparisons between clinical outcomes and gene expression levels. (Months 30 – 36)

PROGRESS: As stated above, we have optimised the NASBA amplification protocol for a number of the biomarkers we have flagged as prostate cancer specific. In addition, we have started assessing samples from 20 patients for expression of these biomarkers. This information will be stored and collated with prospective data collected over the next two years for statistical analysis to draw relevant comparisons between clinical outcomes and gene expression levels.

REPORTABLE OUTCOMES

We have commenced recruitment of patients for this study and have enrolled 44 patients to date. We have increased the number of referral centres and anticipate that our recruitment rate will increase correspondingly.

We have optimised the real-time NASBA amplification technique for individual markers viz, DD3/PCA3 and PSA and are optimising molecular beacons for PSMA and Hepsin. In addition, we are now working to multiplex these reactions.

We have started assessing patient samples with real-time NASBA and have can detect DD3/PCA3 positive cells in ejaculate and post-ejaculate urines from 7 of a total of 20 individuals tested thus far. These results will be analysed statistically, with similar data collected over the next 2 years on additional individuals, and relevant comparisons between clinical outcomes and gene expression patterns will be determined.

CONCLUSIONS

We have modified our research protocol to accommodate the most recent published findings in this field, by expanding our sample collection to include post-DRE urines and utilising the real-time NASBA amplification protocol. We believe that this will strengthen our project aims, and provide valuable additional information to this field of research.

It is our strong expectation that this research will lead to provision of a simple and reliable approach for diagnosing PCa, ultimately reducing the need for the invasive, imprecise and unpleasant procedure of TRUS-guided prostatic biopsies. In addition we expect that the earlier detection will be associated with and even greater proportion of cancer localised to the prostate at time of diagnosis.

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LIST OF APPENDICIES

Appendix 1 – Fradet, Y., F. Saad, et al. (2004). "uPM3, a new molecular urine test for the detection of prostate cancer." *Urology* **64**(2): 311-6

Appendix 2 - Landers, K. A., M. J. Burger, et al. (2005). "Use of multiple biomarkers for a molecular diagnosis of prostate cancer." *Int J Cancer* **114**(6): 950-6.

Appendix 3 – Summary of NASBA amplification with Molecular Beacon Detection from www.basickit-support.com

ELSEVIER

uPM3, A NEW MOLECULAR URINE TEST FOR THE DETECTION OF PROSTATE CANCER

YVES FRADET, FRED SAAD, ARMEN APRIKIAN, JEAN DESSUREAULT, MOSTAFA ELHILALI, CLAUDE TRUDEL, BENOÎT MÂSSE, LYSON PICHÉ, AND CAMILLE CHYPRE

ABSTRACT

Objectives. To evaluate, in a multicenter study, the diagnostic performance of a new molecular test uPM3 for detecting prostate cancer cells in urine because of the need for better methods to identify patients at risk of prostate cancer.

Methods. The uPM3 test is a nucleic acid amplification assay detecting simultaneously in the urine the relative expression of prostate-specific antigen (PSA) mRNA as a marker of prostate cells and PCA3RNA, which is selectively expressed in most types of prostate cancer. The test is performed using the isothermic nucleic acid-based amplification method, and the two targets are simultaneously detected in real-time fluorescence using specific beacons as probes in a thermostated spectrofluorometer. The test was performed on the first voided urine obtained after careful digital rectal examination of the prostate in men undergoing transrectal ultrasound-guided prostate biopsy.

Results. Of 517 patients undergoing biopsy at five centers, 443 (86%) had an assessable sample. Of those, 21%, 55%, and 24% had a total PSA level of less than 4 ng/mL, between 4 and 10 ng/mL, and greater than 10 ng/mL. The corresponding percentage of biopsies positive for cancer in these three groups was 20%, 35%, and 44%. The overall uPM3 sensitivity and specificity was 66% and 89%, respectively. In men with a PSA level less than 4 ng/mL, the sensitivity was 74% and specificity 91%. In those with a PSA level of 4 to 10 ng/mL, the sensitivity was 58% and specificity 91%. In those with a PSA level greater than 10 ng/mL, the sensitivity and specificity was 79% and 80%, respectively. The positive predictive value of uPM3 was 75% compared with 38% for total PSA, and the negative predictive value was 84% compared with 89% and 80% for a PSA cutoff of 2.5 and 4.0 ng/mL, respectively. The overall accuracy was 81% compared with 43% and 47% for total PSA at a cutoff of 2.5 and 4.0 ng/mL, respectively.

Conclusions. These results suggest that the uPM3 molecular urine test may be an important adjunct to current methods for the detection of early prostate cancer. UROLOGY **64:** 311–316, 2004. © 2004 Elsevier Inc.

Prostate cancer is the second leading cause of cancer-related mortality in the Western male population. Serum prostate-specific antigen (PSA) testing and digital rectal examination (DRE) of the prostate have been widely accepted as effective methods of early detection of cancer.[1] However, in 2002, the U.S. Preventive Services Task Force did not recommend routine screening,[2] in good part because of the potential harm resulting from the lack of specificity with current screening methods. In 2 of 3 men with a serum PSA level of 4 ng/mL or greater, biopsies will be negative. However, 20% of those men will have cancer detected if rebiopsied.[3] Furthermore, the rate of cancer in men with a PSA level of 2.5 to 4 ng/mL undergoing systematic biopsies reaches 20% to 23%.[4,5] In screened populations, it has been estimated that approximately one half of the tumors missed in men with a PSA level of 0 to 4 ng/mL had aggressive characteristics and were organ confined.[6] However, lowering the

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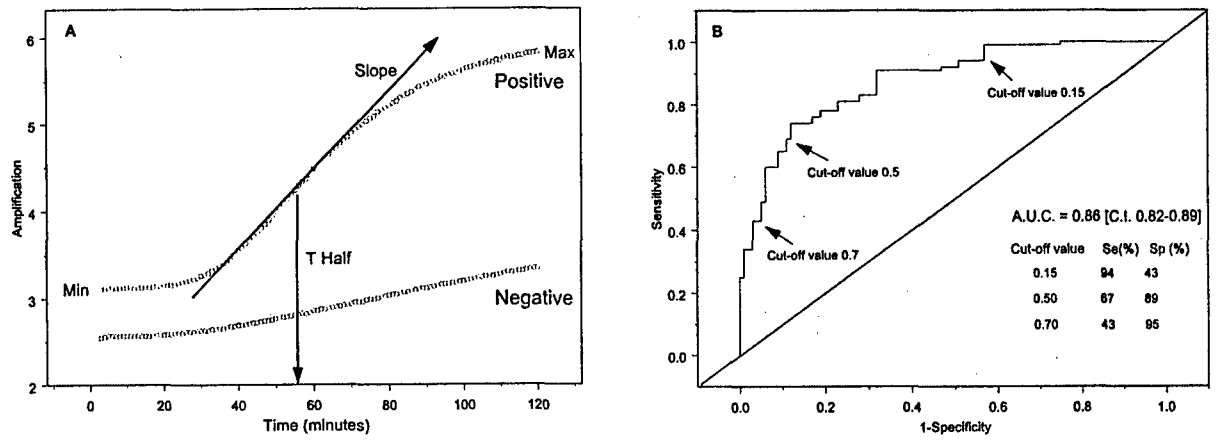

FIGURE 1. *(A) Examples of amplification curves of positive and negative sample showing four parameters measured. (B) Receiver operator characteristic curve of sensitivity and specificity of prediction probability obtained by analysis of amplification curves compared with biopsy results. Cutoff of 0.5 selected for other analysis. A.U.C. = area under curve; C.I. = 95% CI; Se = sensitivity; Sp = specificity.*

threshold for biopsy to 2.5 ng/mL would almost triple the number of men at risk of biopsy and would significantly raise the number of unnecessary biopsies.[7] These dilemmas clearly emphasize the need for more sensitive and specific screening strategies.

To date, only modest improvements in specificity have been observed using tests to measure various forms of free and complexed PSA.[8–10] A promising novel approach is based on the molecular detection of prostate cancer cells in urine obtained after prostatic massage by measuring cancer-specific markers such as GSTP1, telomerase, or $PCA3^{DD3}$ RNA by reverse transcriptase-polymerase chain reaction.[11–13] $PCA3^{DD3}$ is one of the most prostate cancer-specific genes described so far, with overexpression in 95% of cancers tested and a median 66-fold upregulation compared with adjacent non-neoplastic prostatic tissues.[14,15] The quantitative reverse transcriptase-polymerase chain reaction analysis of $PCA3^{DD3}$ gene in urine samples obtained after prostatic massage showed 67% sensitivity and 80% specificity for prostate cancer detection in a recent single-institution study.[13]

We report the results of a multicenter study evaluating the clinical performance of the uPM3 test, a nucleic acid-based amplification assay to measure PSA and $PCA3^{DD3}$ RNA in first voided urine specimens after attentive DRE in 443 patients undergoing transrectal ultrasound-guided prostate biopsy. The study was performed under routine clinical practice conditions and showed a diagnostic accuracy of 81%.

MATERIAL AND METHODS

Urine samples were obtained from 517 men undergoing transrectal ultrasound-guided prostate biopsy at five medical centers. The respective ethical review board of the participating institutions approved the study, and all patients provided written informed consent.

Before transrectal ultrasound-guided biopsy, subjects underwent an attentive DRE, performed by nine different physicians with instruction of doing a thorough prostate palpation for 15 to 30 seconds. After the DRE, the first 20 to 30 mL of voided urine was collected and mixed with an equal volume of phosphate buffer (pH 7.0) and then stored and shipped at 2° to 8°C. The biopsy procedure was not standardized across the five centers, but 6 to 10 cores were taken, depending on the practice of each investigator.

The uPM3 assay was performed in one central laboratory at which the urine samples were processed within 3 days of collection. Spun cells were lysed in a guanidine thiocyanate buffer, and nucleic acid extracts were prepared by the Boom method[16] and eluted in 200 μL DNase/RNase-free water.

Both PSA and $PCA3^{DD3}$ RNA were amplified in the same tube using the nucleic acid sequence-based amplification technology[17] and real-time fluorescence using specific beacon probes to detect the amplification products.[18] The test was performed in duplicates containing 5 μL each of the total nucleic acid extract. The reaction tubes were incubated at 41°C and then read kinetically during a 2-hour period in a thermostated spectrofluorometer (NucliSens Easy Q, BioMerieux, Durham, NC) with the filters set for measurement of fluorescein for $PCA3^{DD3}$ and rhodamine fluorescence for PSA. Figure 1A shows the amplification kinetic curves for a positive and negative PCA3 amplification. The curves were analyzed using a logistic curve fitting routine, including the following four parameters: Max, the upper horizontal asymptote; Min, the lower horizontal asymptote; T Half, the time at the inflexion point; and Slope, a time-scale parameter. Two other parameter estimates were determined: the difference between the asymptotes (Max minus Min) and the ratio of the asymptotes (Max over Min). The six parameter estimates of the $PCA3^{DD3}$ RNA results were used to construct classification trees to predict the outcome of patients (ie, cancer and no

TABLE I. *Positive biopsies versus serum tPSA and DRE categories*

tPSA Range (ng/mL)	Subjects (%)	Positive Biopsies (%)
<4	21 (94/443)	20 (19/94)
DRE suspicious	61 (57/94)	21 (12/57)
DRE negative	39 (37/94)	19 (7/37)
4–10	55 (243/443)	35 (86/243)
DRE suspicious	23 (55/243)	51 (28/55)
DRE negative	77 (188/243)	31 (58/188)
>10	24 (106/443)	44 (47/106)
DRE suspicious	26 (28/106)	68 (19/28)
DRE negative	74 (78/106)	36 (28/78)
Total	100 (443/443)	34 (152/443)

KEY: tPSA = total prostate-specific antigen; DRE = digital rectal examination.

TABLE II. *Sensitivity and specificity of uPM3 by tPSA range*

tPSA Range (ng/mL)	n	Sensitivity (%)	Specificity (%)
<4	94	74 (51–88)	91 (82–95)
4–10	243	58 (48–68)	91 (86–95)
>10	106	79 (65–88)	80 (68–88)
Overall	443	66 (59–74)	89 (85–92)

KEY: tPSA = total prostate-specific antigen.
Data in parentheses are 95% confidence intervals.

cancer on biopsies) according to the serum total PSA (tPSA) range. To confirm the presence of exfoliated prostate cells in the sample, the PSA RNA curve was required to have a Max/Min ratio of at least 1.3 for the sample to be evaluated.

The S-PLUS 2000 software (Insightful, Seattle, Wash) was used to estimate the six parameters of each patient and to construct the classification trees that resulted in a probability of cancer of 0.0 to 1.0. The sensitivity and specificity were computed by comparing the probability outcome predicted by the classification trees and the actual patient biopsy outcomes to construct a receiver operating characteristic curve (Fig. 1B).

Continuous variables, including age and tPSA, were compared between patients with prostate cancer and those with benign conditions using the nonparametric Mann-Whitney *U* test ($P < 0.05$ considered statistically significant). JMP software (SAS Institute, Cary, NC) was used for the computation.

RESULTS

Of the 517 subjects recruited, 443 (86%) provided samples with sufficient PSA RNA signal for analysis of $PCA3^{DD3}$ RNA. The cohort was predominantly white (419 [95%] of 443), with a median age of 64 years (range 40 to 87). Of the 443 men, 152 (34%) had cancer on biopsy. The median tPSA level was 7.5 ng/mL (range 1 to 144) for those with cancer versus 6 ng/mL (range 0.1 to 83) for those without cancer found on biopsy ($P < 0.0001$). Suspicious DRE findings were noted in 48 (32%) of 152 patients with cancer and 55 (19%) of 291 without cancer. The Gleason scores were preponderantly (72%) in the range of 6 to 7 (median 6).

The distribution of subjects according to tPSA level, DRE findings, and percentage of positive biopsies is presented in Table I. The uPM3 test result was a predictive probability obtained by analysis of the amplification curves using a four-parameter logistic curve fitting routine (Fig. 1A) and classification tree. Figure 1B shows the receiver operating characteristic curve computed using these results compared with the actual biopsy outcome. The area under the curve was 0.86 (95% confidence interval [CI] 0.82 to 0.89).

Tables II and III give the clinical performance of the uPM3 urine test according to the serum tPSA values using a cutoff of 0.5 predictive probability. The overall sensitivity was 66%, with a specificity of 89%. The sensitivity and specificity was 74% and 91% for a PSA level less than 4 ng/mL, 58% and 91% for a PSA level of 4 to 10 ng/mL, and 79% and 80% for a PSA level greater than 10 ng/mL, respectively. No statistically significant difference was found in the sensitivity or specificity of the uPM3 assay in patients with or without suspicious DRE findings. The positive predictive value (PPV) for the uPM3 test was 75% versus ~40% for tPSA. The negative predictive value (NPV) was equivalent between the two tests, but, because of the greater PPV for uPM3, the accuracy of the uPM3 test was nearly twofold greater than tPSA determination (81% versus 43% and 47% for tPSA cutoffs of 2.5 and 4 ng/mL, respectively).

Of the total population of 443 assessable men, 91 underwent subsequent biopsy after one or more previous negative biopsies. The performance of the uPM3 test in this subset was similar to that in the overall cohort, with a sensitivity of 74% (95% CI 57% to 86%) and a specificity of 87% (95% CI 76% to 93%) corresponding to an NPV of 87% (95% CI 76% to 93%) and a PPV of 74% (95% CI 57% to 86%).

COMMENT

The widespread acceptance of serum PSA testing for the early detection of prostate cancer has been hampered by the low specificity of the assay and the resulting cost and anxiety generated by unnecessary biopsies. The problem is further complicated by the results of recent studies showing that almost 1 in 4 men with a PSA level of 2.5 to 4 ng/mL have prostate cancer on biopsy,[4,5] of which more than one half are aggressive cancers that are

TABLE III. *uPM3 performance versus serum tPSA level*

Variable	PPV (%)	NPV (%)	Accuracy (%)
tPSA ≥2.5 ng/mL	37 (33–42)	89 (77–95)	43 (39–48)
tPSA ≥4 ng/mL	38 (33–43)	80 (71–87)	47 (42–52)
uPM3	75 (67–82)	84 (79–87)	81 (77–84)

KEY: tPSA = total prostate-specific antigen.
Data in parentheses are 95% confidence intervals.

organ confined and thus within the appropriate window of opportunity for cure.[6] Large-scale randomized prostate cancer screening trials have shown that approximately 20% of men have a PSA level between 2 and 4 ng/mL, stressing the important burden that would result from lowering the PSA threshold for prostate biopsy.[7] The results of the present study have shown that the uPM3 urine test may represent a statistically significant improvement, with an overall accuracy twofold greater than that of the tPSA assay. A salient feature of this assay was its high specificity, which averaged 89% in the 443 patients tested. The specificity was slightly lower in men with a PSA level greater than 10 ng/mL, but such men have the greatest probability of cancer on repeat biopsy. The NPV was similar to that of the tPSA assay, but a positive uPM3 test was associated with a positive biopsy 75% of the time, twice the PPV of tPSA measurement. Because the test is the equivalent of molecular cytology of the prostate, it was interesting to note that individuals with a positive uPM3 test may be at greater risk of cancer than those with prostate biopsy features considered to be high risk such as high-grade prostatic intraepithelial neoplasia and atypia, which have a PPV for cancer on repeat biopsies varying from 10% to 50% depending on the study.[19–21] Only 1 of 14 with high-grade prostatic intraepithelial neoplasia and 0 of 6 with atypia in the present study had a positive uPM3 test.

The performance of the uPM3 test in this multicenter setting was very similar to that of previously reported single-institution research assays.[11–13] In previous reports, the assays were performed after prostatic massage that was described as extensive in at least one study.[13] In the present trial, nine different physicians performed what was requested to be an attentive DRE but was not standardized. It is possible that a true prostatic massage would have increased the cell yield and thus the sensitivity of cancer detection. The test was usable in only 443 (86%) of the 517 patients tested because of the lack of detection of prostate cells. It is also possible that additional technical improvements in the assay may increase its sensitivity in the future. Others have shown, in a different type of assay, an improvement of 36% to 100% in the detection of cancer in urine after prostatic massage by changing the kit used for RNA isolation.[11,22] All biopsies performed in our study were ultrasound guided, but the number of cores taken varied among centers from 6 to 8 or 10. Uncertainties remain as to the optimal biopsy protocol, because the only randomized trial showed no improvement in cancer detection by increasing the number of cores from 6 to 12.[23] Nevertheless, the frequency of positive biopsies in our study was very similar to that reported in contemporary series. The performance of the uPM3 test was similar in men with suspicious DRE findings or negative DRE findings.

One of the most promising characteristics of the uPM3 test was its high accuracy in the 94 men with a PSA level less than 4 ng/mL. In this range, the uPM3 test had 74% sensitivity and 91% specificity. This type of noninvasive test may be particularly attractive to identify those at high risk of cancer among the large population of men with a PSA level between 2.5 and 4 ng/mL. Another significant challenge in current practice is to determine who should undergo repeat biopsy and how often. The uPM3 test also performed very well in the 91 subjects undergoing subsequent biopsies after one or more previous negative biopsies. In this subset, the uPM3 test had 74% sensitivity and 87% specificity, corresponding to an NPV of 87% and a PPV of 74%. On the basis of the limited data from the present study, the PPV of the test may be better than commonly used risk factors such as prostatic intraepithelial neoplasia or atypia. No information was available on the extent of cancer in the present study. Future investigations should address the potential prognostic value of the uPM3 test to determine cancer aggressiveness.

The high specificity of the uPM3 test was likely a result of the very high discriminating power of the gene expression in prostatic cancer cells. In real-time quantitative reverse transcriptase-polymerase chain reaction studies performed on radical prostatectomy specimens, the median expression of $PCA3^{DD3}$ in tumor tissues was 5849 normalized

mRNA copies versus 10 for the telomerase reverse-transcriptase (hTRT) gene.[15] The same group recently showed a median 11-fold upregulation in 13 prostatic tissue samples containing less than 10% of tumor cells, suggesting that nucleic acid amplification testing using the $PCA3^{DD3}$ gene is capable of detecting very few malignant cells in a background of predominantly nonmalignant prostatic cells.[13] Because of the nature of this type of assay in detecting rare events, it was not possible to determine the robustness of the test in individual patients. The results of the present study will need to be validated in other clinical trials that should also aim at elucidating the relationship of the test result with the extent of cancer, as well as the possible causes of false-positive and false-negative results.

CONCLUSIONS

The nucleic acid amplification test uPM3 to detect PSA and $PCA3^{DD3}$ RNA in voided urine after DRE performed well in routine clinical settings and was highly specific. With an NPV comparable to that of the PSA assay, the overall accuracy of the uPM3 test was 81% compared with approximately 40% for tPSA. The uPM3 test may provide some answers to the current dilemmas of early prostate cancer detection and may be particularly useful in the monitoring of men with lower PSA values and those with previously negative biopsies.

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EDITORIAL COMMENT

In this report, Fradet *et al.*, present the results of a multicenter study on the diagnostic value of the first gene-based test for prostate cancer.

The uPM3 test measures the expression of the $PCA3^{DD3}$ gene, which was identified by Bussemakers and colleagues.[1] The first exploratory study by Hessels *et al.*[2] indicated that gene-based testing for the diagnosis of prostate cancer was feasible and that the use of the relatively novel substrate (ie, urinary sediment after extended DRE), was very promising. Fradet *et al.* now provide additional evidence that the uPM3 test can predict the presence of prostate cancer with extremely high accuracy. This will have profound additional value within the PSA range of 4 to 10 ng/mL, because of very high

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Use of multiple biomarkers for a molecular diagnosis of prostate cancer

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The identification of biomarkers capable of providing a reliable molecular diagnostic test for prostate cancer (PCa) is highly desirable clinically. We describe here 4 biomarkers, *UDP-N-Acetyl-α-D-galactosamine transferase* (*GalNAc-T3*; not previously associated with PCa), *PSMA*, *Hepsin* and *DD3/PCA3*, which, in combination, distinguish prostate cancer from benign prostate hyperplasia (BPH). *GalNAc-T3* was identified as overexpressed in PCa tissues by microarray analysis, confirmed by quantitative real-time PCR and shown immunohistochemically to be localised to prostate epithelial cells with higher expression in malignant cells. Real-time quantitative PCR analysis across 21 PCa and 34 BPH tissues showed 4.6-fold overexpression of *GalNAc-T3* (p = 0.005). The noncoding mRNA (*DD3/PCA3*) was overexpressed 140-fold (p = 0.007) in the cancer samples compared to BPH tissues. *Hepsin* was overexpressed 21-fold (p = 0.049, whereas the overexpression for *PSMA* was 66-fold (p = 0.047). When the gene expression data for these 4 biomarkers was combined in a logistic regression model, a predictive index was obtained that distinguished 100% of the PCa samples from all of the BPH samples. Therefore, combining these genes in a real-time PCR assay represents a powerful new approach to diagnosing PCa by molecular profiling. (Supplemental material for this article can be found on the *International Journal of Cancer* website at http://www.interscience.wiley.com/jpages/0020-7136/suppmat/index.html.)

Key words: diagnosis; prostate cancer; PSMA; DD3/PCA3; Hepsin; multivariate analysis

Adenocarcinoma of the prostate (PCa) is the most common internal malignancy diagnosed in men and the second most common cause of male cancer deaths in the western world.[1,2] Current methods employed in PCa diagnosis include digital rectal examination (DRE) and serum prostate-specific antigen (PSA) testing followed by transrectal ultrasound (TRUS)-guided biopsies.

Although PSA is used erroneously to indicate a diagnosis of PCa, elevated PSA levels are also caused by the very common conditions of prostatitis and benign prostatic hyperplasia (BPH),[3] significantly reducing its specificity and limiting its diagnostic value.[4,5] Furthermore, TRUS-guided prostate biopsies are imprecise, even when used in conjunction with adaptations to PSA (such as percentage-free PSA) and ultrasound imaging,[7] necessitating a need for multiple (>6 and preferably ≥8) cores to be taken to minimise false-negative rates.[6] A further limitation is that, even with earlier diagnosis afforded by the current 2-step PSA/TRUS biopsy approach, approximately 25% of patients already have metastatic disease.[8]

The time-honoured quest for reliable prostate cancer markers to complement established histopathologic techniques to identify cancer cells in blood, bone marrow, urine, prostatic tissue itself and our area of interest, semen, has embraced a number of protein markers in addition to PSA. Prostate-specific membrane antigen (PSMA) has been detected in both benign tissue and PCa and in clinical terms is generally regarded as prostate-specific.[9] Hepsin, a membrane-bound serine protease, which, although widely expressed throughout the body, is upregulated in malignant prostatic epithelium[10–12] and expression of both *PSMA* and *Hepsin* have also been demonstrated to be upregulated by cDNA profiling of cancers[13,14] compared to BPH. This approach has identified many genes for which expression patterns are altered in malignant tissues. One such gene is *DD3/PCA3*, which is a noncoding mRNA transcript, with one specific splice variant highly overexpressed in PCas.[15]

Recently the focus of some studies has changed from identification of individual markers to utilising combinations of known PCa-specific markers as predictors of disease recurrence after treatment with curative intent.[16,17]

In our study, we show that a novel gene, *UDP-N-Acetyl-α-D-galactosamine transferase* (*GalNAC-T3*), in combination with 3 other candidate genes, is highly predictive in the diagnosis of PCa.

Material and methods

Specimen collection

All tissue specimens were obtained from consenting patients at the Royal Brisbane Hospital, Queensland, as approved by the Ethics Committee of that hospital. Tissue sections were collected and processed as previously described.[14] Specimens were chosen for analysis based on histologic evaluation by H&E staining, demonstrating that the samples contained predominantly benign epithelial cells (in the case of BPH samples) of comparable proportions to adenocarcinoma cells (in the case of PCa samples).

Laboratory analyses

RNA was extracted from frozen tissues (collected as described above) and reverse transcribed as previously described.[14] Three genes previously identified by microarray profiling[14] and 8 candidate genes were screened against PCa (21) and BPH (35) samples using the Rotorgene Real-time PCR machine (Corbett Research, Sydney, Australia). Primers for these genes are outlined in Supplementary Table 1. (Supplemental material for this article can be found on the *International Journal of Cancer* website at http://www.interscience.wiley.com/jpages/0020-7136/suppmat/index.html.) Each marker was analysed against a standard curve determined by expression of the housekeeping gene β_2-*microglobulin* in the normal prostate epithelial HPV-18 transformed cell line PZ-HPV-7.[18] Each sample was analysed in duplicate within a minimum of 2 independent experiments. The expression of the markers of interest was normalised against the housekeeping gene (β_2-*microglobulin*) for each replicate tissue sample. The ratios of gene expression/10^5 copies of β_2-*microglobulin* were calculated, in samples of PCa and BPH tissues, using EXCEL (Microsoft Corporation, Redmond, WA) software. The values for each sample were normalised to the 50th percentile within each experiment and then averaged across the experiments and plotted as log-transformed ratios.

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As GALNT3 expression has not previously been described in prostate tissues, immunohistochemistry was performed on frozen sections to determine epithelial cell localisation, using an anti-GALNT3 polyclonal antibody, kindly donated by Professor Kimitoshi Kohno from the Department of Molecular Biology and Surgery, University of Occupational and Environmental Health, Japan, School of Medicine, Kitakyushu, as previously described.[19] The sections were scored based on the intensity of the colour development and number of the cells within the sample that were positive.

Statistical analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS; version 10.00; SPSS Inc., Chicago, IL). Mean levels of biomarker expression were compared between PCa and BPH patients using 2-tailed *t*-tests. Logistic regression analysis was used as the basis of determining which combination of markers best predicted a clinical diagnosis. The predictive equation (from the logistic model) was used to describe the expected probability of having clinically diagnosed PCa. The probability of having PCa $= 1/\{1+ \mathrm{Exp}[-(\beta_0 + \Sigma(\beta_i \times Q_i))]\}$, where β_0 is a constant, β_i is the predictive regression coefficient value for each biomarker and Q_i is the level of expression.[20] The regression model assigns a coefficient to each biomarker (β_i), and this coefficient weights each biomarker according to its importance in determining each individual's risks of clinical diagnosis. The predicted probability of PCa based on the regression equation was used to classify patients as either cancer (predicted value >0.5) or BPH (<0.5), and the accuracy of the model was assessed as the percentage of patients who were correctly classified. Only biomarkers with a *p*-value of less than 0.05 were used in the final predictive equation, and 95% confidence intervals for percentages were calculated using the exact binomial method.

Results

Candidate marker selection

To establish a set of biomarkers with greater predictive value for the diagnosis of PCa than PSA, we selected a number of gene markers overexpressed in malignant prostate tissues, cancer-associated Sm-like gene (CaSm), UDP-*N*-Acetyl-α-D-galactosamine transferase 3 (Gal*N*Ac-T3) and protein tyrosine phosphatase nonreceptor type 3 (PTPN3), identified in microarray profiling experiments. We also selected a set of candidate genes, PSMA,[14] Hepsin,[13] DD3/PCA3,[15] PLA2,[14] PB39,[14] *PCTA-1*,[21] *VEGFA*[22] and *HPC2*[23] previously identified by ourselves and other groups (Table I).

The expression levels of the candidate marker genes, described above, were confirmed using quantitative real-time PCR analysis of PCa and BPH tissues. All genes were standardized against the housekeeping gene β_2-microglobulin to confirm their differential expression patterns in cancer and BPH samples. These cancer and BPH specimens were collected and classified according to the histopathologic diagnosis of the tissues removed during surgery, and a percentage of the amount of cancer present in each fragment was provided by a pathologist with particular expertise in uropathology (Supplementary Table 2).

Since there was no significant difference in mRNA expression between 5 tumors and 5 BPH samples for *CaSm* ($p = 0.326$), *HPC2* ($p = 0.257$) and *PCTA-1* ($p = 0.137$), these genes were considered not to be useful as biomarkers for PCa diagnosis (Tables I and II). After analysis of larger sample groups, *VEGFA* (10 PCa and 14 BPH samples; $p = 0.139$), PB39 (15 PCa and 17 BPH samples; $p = 0.063$), *PLA2* (11 PCa and 16 BPH samples; $p = 0.285$) and *PTPN3* (8 PCa and 9 BPH samples; $p = 0.099$) also failed to discriminate significantly between BPH and PCa (Table II).

Conversely, when the transcription ratio to β_2-microglobulin was compared for 4 other biomarkers (*GalNAc-T3*, *PSMA*, *Hepsin* and *DD3/PCA3*), no overlap of the sample group means was observed. Only 1 of the genes identified in the microarrays, *GalNAc-T3*, showed a significant difference ($p = 0.005$) between the tumors (mean = 3.97; range 0.22–15) and BPH (mean = 0.85; range 0.03–3) groups with a 4.6-fold overexpression in the real-time PCR assay (Table II; Fig. 1). Analysis of 3 candidate genes revealed highly significant differences between the 2 groups of patients (Table II; Fig. 1). The non-coding mRNA (DD3/PCA3) was overexpressed 140-fold ($p = 0.007$) in the cancer samples (mean = 5.65; range 0–28) compared to BPH tissues (mean = 0.04; range 0–0.7). *Hepsin*, encoding a transmembrane serine protease, was overexpressed 21-fold ($p = 0.049$; cancer, mean = 13.12, range 0.4–113; BPH, mean = 0.62, range 0.02–2.7), whereas the overexpression for PSMA was 66-fold ($p = 0.047$; cancer, mean = 35.88, range 0.8–260; BPH, mean = 0.54, range 0.01–2.12).

GalNAc-T3 localization

As GalNAc-T3 has not been previously associated with PCa, we verified that this gene was epithelially expressed by immunohistochemistry with an anti Gal*N*Ac-T3 antibody on 5 benign and 5 tumor frozen tissue sections. A Gleason 3+4 sample (Table III; Fig. 2) showed strong staining of the malignant epithelium. High-

TABLE I – CANDIDATE GENE AND MICROARRAY SUMMARY

	Gene function	Gene expression ratios (test samples/reference)		Fold increase in expression detected by arrays	Reference no.
		BPH	PCa (Av)		
Markers identified by arrays					
CaSm (LSM1)	Pre-mRNA splicing; mRNA degradation	0.225	0.9, 1.09, 1.28 (1.09)	4.6	40
GalNAc-T3 (GALNT3)	Catalyst for mucin type-O glycosylation	0.2	1.25, 0.95, 1.05 (1.09)	5.2	41
PTPN3	Protein tyrosine phosphatase	ND	1.2, 0.85, 1.14 (1.06)	100	42
Candidates					
Hepsin	Transmembrane serine protease				13
PLA2	Phospholipase A2 group IIA-inflammatory response regulator				14
DD3/PCA3/PCA3	Non-coding mRNA				15
POV1PB39	Gene of unknown function-overexpressed in prostate cancer				14
HPC2	Metal dependent hydroxylase				23
PCTA-1	Related to human galectin-8 gene				21
VEGFA	Angiogenic cytokine				43
PSMA (FOLH1)	Prostate-specific membrane antigen				14
β_2-microglobulin	Housekeeping gene				44

TABLE II – QUANTITATIVE PCR SUMMARY

Gene	Sample groups	Sample no. number	Gene expression/ β2-microglobulin	Standard error	*t*-test significance
GalNAc-T3	PCa	20	3.97	0.98	0.005
	BPH	35	0.85	0.12	
DD3	PCa	18	5.65	1.83	0.007
	BPH	34	0.04	0.20	
PSMA	PCa	20	35.88	16.62	0.047
	BPH	31	0.54	0.08	
Hepsin	PCa	20	13.12	5.94	0.049
	BPH	35	0.62	0.12	
PB39/POV1	PCa	15	20.40	23.52	0.063
	BPH	17	1.66	1.60	
PTPN3	PCa	8	2.29	1.41	0.099
	BPH	9	1.02	0.80	
PCTA-1	PCa	5	4.26	2.85	0.137
	BPH	5	0.93	0.52	
VEGFA	PCa	10	4.73	6.43	0.139
	BPH	14	0.45	0.38	
PSA	PCa	11	5.28	5.32	0.152
	BPH	12	0.87	0.52	
HPC2	PCa	5	1.90	0.75	0.257
	BPH	5	1.03	0.86	
PLA2	PCa	11	24.12	33.24	0.285
	BPH	16	1.13	1.35	
CaSm	PCa	6	1.92	1.35	0.326
	BPH	5	1.06	0.37	

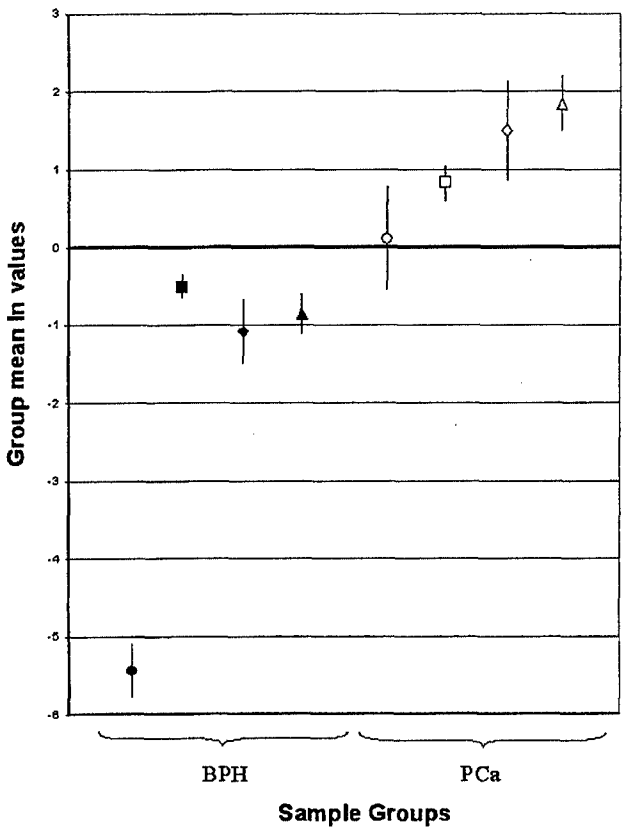

FIGURE 1 – Graph of the sample group means (samples expressed as log-transformed ratios) for each biomarker. BPH sample group: DD3, closed circles; *GalNAc-T3*, closed squares; *Hepsin*, closed diamonds; *PSMA*, closed triangles. Cancer samples: *DD3*, open circles; *GalNAc-T3*, open squares; *Hepsin*, open diamonds; *PSMA*, open triangles. Error bars indicate ± S.E. for each biomarker.

grade PIN also showed intense staining of epithelial nuclei (Table III; Figure 2). The BPH samples showed negative to weak staining of benign atrophic glands. The intensity of the stain was found to increase with the increase in Gleason scores or the higher-grade cancers (Table III).

Use of combined biomarkers for PCa diagnosis

It was possible to discriminate between the 2 groups with each of the 4 markers described above using univariate analysis. However, since there was considerable variation of gene expression within the sample groups, the use of a single marker to diagnose PCa in individuals would not be sufficiently discriminating. A combination of markers would be expected to enhance the diagnostic power of the assay. Using a logistic regression model,[20] we determined whether all 4 markers, combinations of 3 markers from this set, or the use of single markers were most useful in establishing a difference between cancer and BPH. Initially, we analyzed a training set of randomly selected cancer (n = 8) and BPH (n = 20) samples using *DD3/PCA3*, *Hepsin* and *PSMA*, to establish a predictive equation. Application of this equation gave 100% correct prediction for all of the individuals in both categories. When the same equation was employed to the other cancer (n = 13) and BPH (n = 15) samples, it correctly identified 100% of the cancers and 80% of the individuals with BPH.

Analysis of the complete set of data using all 4 markers, *GalNAc-T3*, *PSMA*, *Hepsin* and *DD3/PCA3*, predicted the classification of 100% of all samples correctly (Table IV; Fig. 3). When *DD3/PCA3* was omitted from the analysis, the equation had 91% accuracy in predicting the sample designations. In the absence of *PSMA*, the accuracy of the test fell to 96%, whereas leaving out *Hepsin* also reduced the accuracy to 96%. When GalNAc-T3 was omitted, the predictions were 100% correct. Therefore, the use of *DD3/PCA3*, *Hepsin* and *PSMA* was chosen as the best multivariate predictive model.

Conversely, when each biomarker was used alone in a predictive regression model, *DD3/PCA3*, *PSMA* and *Hepsin* showed reduced accuracy in distinguishing BPH from cancers. *DD3/PCA3* alone had 90% accuracy; *PSMA* showed 81% accuracy, whereas *Hepsin* had 80% accuracy in distinguishing PCa from BPH.

TABLE III – IMMUNOSTAINING RESULTS FOR GALNT3

Sample	Cellular structures identified within the sections stained	Intensity of stain	Gleason score of tissue section
B01-00	Prostatic urothelium	+	–
B04-00	Benign glands	–	–
B05-00	Benign atrophic glands	+	–
B07-00	Benign atrophic glands	–	–
B013-00	Benign glands	–	–
C05-00	Malignant tissue	++++	3 + 4
C09-00	Malignant tissue and benign glands	+/++	4 + 5
C11-00	High-grade PIN & malignant tissue	+++	4 + 3
C15-00	Benign glands & malignant tissue	+	4 + 3
C22-00	High-grade PIN/noninvasive cancer	+++	3 + 4

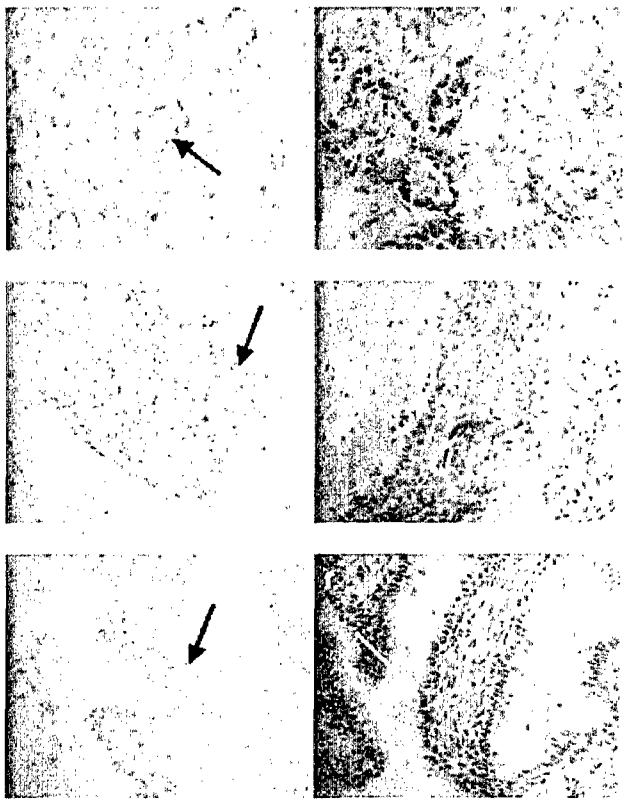

FIGURE 2 – Immunohistochemical determination of GALNT3 expression in tumor and BPH tissue. *(a)* High-grade cancer sample C05-00 stained strongly in the epithelial nuclei (40×); *(b)* Hemotoxylin and eosin (H&E) section of C05-00 (Gleason score 3+4; 40×); *(c)* Epithelial nuclei also stained in the high-grade prostatic intraepithelial neoplastic (PIN) sample, C22-00 (40×); *(d)* H&E section of C22-00 (40×); *(e)* No staining found in the epithelial cells of the BPH sample, B13-00 (40×); *(f)* H&E section of B13-00 (40×). Arrows indicate epithelial glands found in each section. The brown stain is representative of Gal*N*Ac-T3 expression.

Discussion

Curative treatment for PCa is only possible for localized disease, which is normally identified by investigation of an elevated serum PSA level. Although the majority of patients diagnosed present with PSA levels from 4 to 10 ng/ml, only 25% of men with PSA levels in this range will have a diagnosis of PCa.[24] This is the case even when as many as 12 TRUS-guided biopsies are taken as has been recently advocated.[6] A further confounding factor for the PSA/TRUS approach is the observation that a small proportion of more aggressive dedifferentiated tumors are less often accompanied by PSA elevation in serum.[25]

Attempts to combine PSA levels, clinical stage and Gleason score to predict pathologic stage for men with clinically localized PCa at the time of diagnosis are imprecise with all 3 parameters being late consequences of multiple abnormalities at a molecular level.[1, 26] The development of new more predictive markers to assist in early detection and prognosis is highly desirable. In our study, we identified a new potential PCa-specific marker GALNT3, which is involved in initiating mucin-type O-glycosylation by catalyzing the formation of an O-glycosidic link between Gal*N*Ac and serine or threonine residues. Previous studies have revealed GALNT3 is only expressed in organs that contain secretory epithelia and is highly expressed in human tumor cell lines arising from epithelial glands.[20,27] In addition, increased GALNT3 expression was associated with moderately and poorly differentiated tumors in small cell lung carcinomas and with poorer prognostic outcomes,[28] similar to our findings in PCa.

We identified a significant ($p = 0.005$) increase in *GalNAc-T3* expression in PCa tissues through gene expression profiling using quantitative real-time PCR. Immunohistochemical results correlated with the localization of GALNT3 in epithelial cells as previously reported,[20,29] with greater expression in malignant tissue sections compared to BPH sections and increased staining intensity corresponding with increasing Gleason score of tissue sections.

Recent reports have cited the use of single molecular markers in the diagnosis and prognosis of PCa.[30–32] Although all of these individual markers have been able to discriminate between cancer and benign prostatic hyperplasia (BPH) as sample groups, none is sufficient for predicting the presence of PCa in individual patients with total reliability.[33]

We have demonstrated here that a combination of *DD3/PCA3*, *GalNAc-T3*, *Hepsin* and *PSMA* is highly predictive in distinguishing between PCa and BPH. The predictive ability of the logistic regression model decreased markedly when only single biomarkers were considered, which is in keeping with published results for single markers.[30–32] Several other models such as neural networks and tree-based methods have been proposed for predictive analysis of biomarkers and clinical outcomes. However, a recent conference relating to innovations and challenges in PCa research concluded that these other models do not provide superior accuracy when compared to the traditional regression analysis, which supplies insight into hazard ratios and *p*-values for predictors.[34] Therefore, nomograms based on logistic regression models appear to maximize predictive accuracy.[34]

It is evident that these markers (above) have varied roles in normal cellular processes reflecting multiple molecular aberrations associated with the malignant phenotype. PSMA, also known as glutamate carboxypepidase II, is an enzyme that digests polyglutamyl folates to monoglutamyl folates.[35] Although the precise role PSMA plays in tumorigenesis has not been defined, it is clear that

TABLE IV – STATISTICAL ANALYSIS OF BIOMARKERS

Marker combinations	Sample groups	Sample nos.	Percent of samples correctly categorised (95% CI)
Multivariate analysis			
GalNAc-T3	PCa	18	100.0% (81.5–100)
PSMA	BPH	32	100.0% (89.1–100)
Hepsin	Overall	50	100.0% (92.9–100)
DD3			
Hepsin	PCa	18	100.0% (81.5–100)
PSMA	BPH	32	100.0% (89.1–100)
DD3	Overall	50	100.0% (92.9–100)
GalNAc-T3	PCa	18	94.4% (72.7–99.9)
Hepsin	BPH	34	97.1% (84.7–99.9)
DD3	Overall	52	96.2% (86.8–99.5)
GalNAc-T3	PCa	18	94.4% (72.7–99.9)
PSMA	BPH	33	97.0% (84.2–99.9)
DD3	Overall	51	96.1% (86.5–99.5)
GalNAc-T3	PCa	21	81.0% (58.1–94.6)
Hepsin	BPH	33	97.0% (84.2–99.9)
PSMA	Overall	54	90.6% (79.7–96.9)
Univariate analysis			
DD3	PCa	18	77.8% (52.4–93.6)
	BPH	35	97.1% (85.1–99.9)
	Overall	53	90.6% (79.3–96.9)
PSMA	PCa	21	47.6% (25.7–70.2)
	BPH	34	94.1% (80.3–99.3)
	Overall	55	76.5% (63.0–86.8)
Hepsin	PCa	21	71.4% (47.8–88.7)
	BPH	35	91.4% (76.9–98.2)
	Overall	56	83.9% (72.6–91.8)

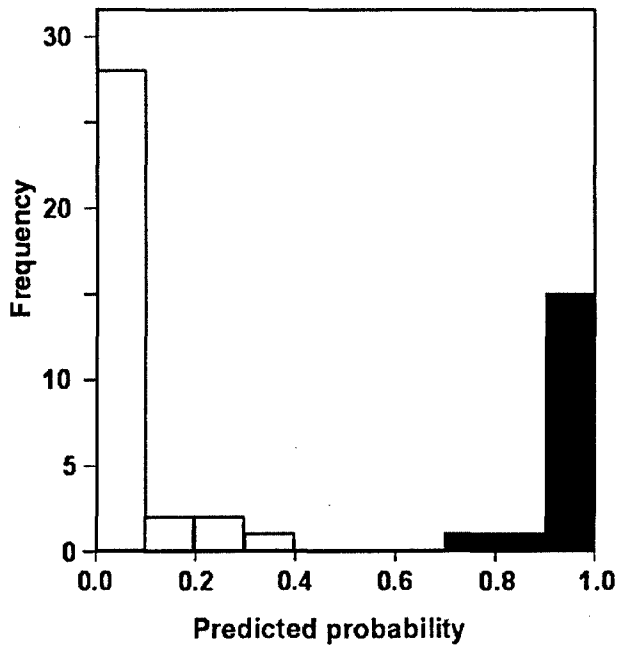

FIGURE 3 – Histogram showing predicted probability of individual samples containing PCa (= 1; closed bars) or BPH (= 0; open bars) tissue based gene expression ratios for PSMA, DD3 and Hepsin using the logistic regression model. Total sample size = 50; mean = 3.600; S.D. = 0.4566661.

it is important for cellular metabolism and growth and is associated with tumor neovasculature.[36] Hepsin is a member of the serine protease family, which are single- or 2-chain zymogenes that are activated by specific and limited proteolytic cleavage.[37] These enzymes are involved in processes such as blood coagulation, fibrinolysis and complement activation, and most recently have been implicated in vascular function and angiogenesis.[38] *DD3/PCA3* was identified by using differential display analysis to determine mRNA expression patterns of normal compared to tumor tissue from the human prostate.[15] As no extensive open reading frame could be found, it has been proposed that *DD3/PCA3* may function as a non-coding RNA. Non-coding RNAs have been shown to control chromosome architecture, mRNA turnover and the developmental timing of protein expression and may also regulate transcription and alternative splicing.[39] To date it is unclear how these genes contribute to the development of tumorigenesis.

The value of the regression model and its application is that it does not rely on specific functions of these biomarkers being assessed. Application of this methodology is clearly not confined to the set of 4 biomarkers described in this article. Previous results from a variety of laboratories have identified other markers that are overexpressed in PCa. Indeed we have previously identified other candidate markers including δ-catenin, an adhesive junction associated protein, and NEK3, a serine protein kinase, which may also have utility in this regard.[14] However, regardless of the number of markers, limitations exist in this methodology that include heterogeneity of tumour samples, amount of tumour present and quality of RNA and cDNA generated.

In our study, we have shown that a combination of markers greatly enhances sensitivity of molecular diagnosis of PCa compared to single marker identification using histopathologic characterisation of tissues as a reference. The relevance of any

new marker is indicated by its ability to differentiate cancer from non-cancer translating into an improvement in diagnostic accuracy.[33] As our experience with single molecular markers used to evaluate tissue sections and enriched cells from urine and ejaculate is consistent with those of other authors,[31,45] we plan to proceed to apply the principle illustrated by our study and examine multiple markers in enriched prostate cells as an adjunct to TRUS-guided biopsies in prostate cancer diagnosis.

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NASBA and Molecular Beacons: The Perfect Match of Technologies

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Introduction

Nucleic acid amplification technologies, such as PCR and NASBA, have become powerful investigative tools for scientists from a wide range of disciplines. Although these technologies were first applied to areas of basic research, they are now routinely utilized in the clinical diagnostic laboratory. In particular, these methods have proven to be both highly accurate and sensitive for the molecular diagnosis of various infectious diseases.

The most common way to evaluate the outcome of an amplification reaction is to analyze the end products by conventional detection procedures such as gel electrophoresis or by means of specific probe based detection technologies.The inherent disadvantage of these detection methods is that they require additional handling of amplified products. This step has the potential risk of contaminating lab equipment or lab areas with amplicons that can serve as new templates in subsequent experiments, thereby leading to false positive results.Ideally, the detection of amplification product generated during a reaction should be monitored "real time" while the reaction proceeds in a single closed tube.

bioMérieux has now combined two state-of-the art technologies - NASBA amplification and Molecular Beacon based probe detection - for a new real time diagnostic test system called NucliSens EasyQ. The NucliSens EasyQ simple test format was designed to significantly reduce technical hands-on-time and to provide rapid results, thus meeting the needs of the routine clinical laboratory for future NAD testing.

NASBA Amplification

NASBA is an isothermal, nucleic acid amplification technology that is based on the concerted action of three enzymes - AMV-Reverse Transcriptase, RNase-H and T7-RNA Polymerase. In combination with target specific oligonucleotide primers it amplifies RNA targets (and also DNA targets in a modified procedure) to greater than a billion fold in 90 minutes reaction time. The amplification reaction proceeds at 41°C and, importantly, generates single stranded RNA molecules as end-product (see www.nuclisens.com for more detailed information on the mode of action).

Molecular Beacons

Molecular Beacons (MB) represent a novel class of hybridization probes that fluoresce upon hybridization in solution to a complementary target sequence. MB

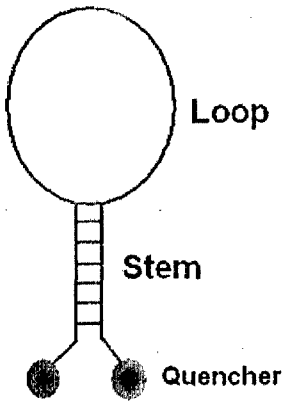

Figure 1. Structure of Molecular Beacon probe.

probes were first described in 1996 by Sanjay Tyagi and Fred Russell Kramer (see ref 1), and have been used for a variety of applications. MB probes are short nucleotide sequences consisting of a stem-loop structure containing a sequence complimentary to the target sequence with a fluorophore and a quencher molecule at the respective ends (see figure 1). In their normal state the stem keeps the fluorophore and the quencher in close proximity to each other thereby preventing emission of fluorescence. In the presence however of a target sequence that is complimentary to the loop sequence, the probe hybridizes and undergoes a conformational change resulting in unfolding. In this state the quencher can no longer absorb photons emitted by the fluorophore and the probe starts to fluoresce upon excitation. Importantly, molecular beacons can be labeled with different fluorescent molecules and this allows co-amplification and simultaneous co-detection of different amplification reactions in single tube.This is an important feature in creating a quantitative test based on co-amplification of a "wild-type" target in combination with an internal calibrator RNA molecule (see figure 2).

The Perfect Match of Technologies

To create a real time NASBA based amplification procedure, NASBA has now been combined with molecular beacon based fluorescent detection. The strengths of both technologies - the generation of single stranded RNA molecules by isothermal NASBA amplification and the ability of molecular beacons to hybridize to single stranded molecules - are a perfect match. In a single tube the NASBA reaction generates single stranded RNA molecules to which target specific molecular beacon probes bind simultaneously. The formation of the newly generated RNA molecules is thus determined real time by continuous monitoring of fluorescence in a dedicated fluorescent Analyzer. For a typical assay, the reaction time for real time amplification and detection is just one hour.

NucliSens EasyQ

NASBA amplification and real time detection with molecular beacons are combined in a new product line called NucliSens EasyQ. The first NucliSens EasyQ product on the market in early 2002 will be a Quantitative HIV-1 Assay to be used for viral load monitoring in plasma samples. NucliSens EasyQ will encompass reagents as well a new, dedicated fluorescent Analyzer, intuitive software for data reduction and automated test result reporting. In the next issue of Sensor the features and characteristics of this novel tool for HIV-1 viral load monitoring will be outlined.

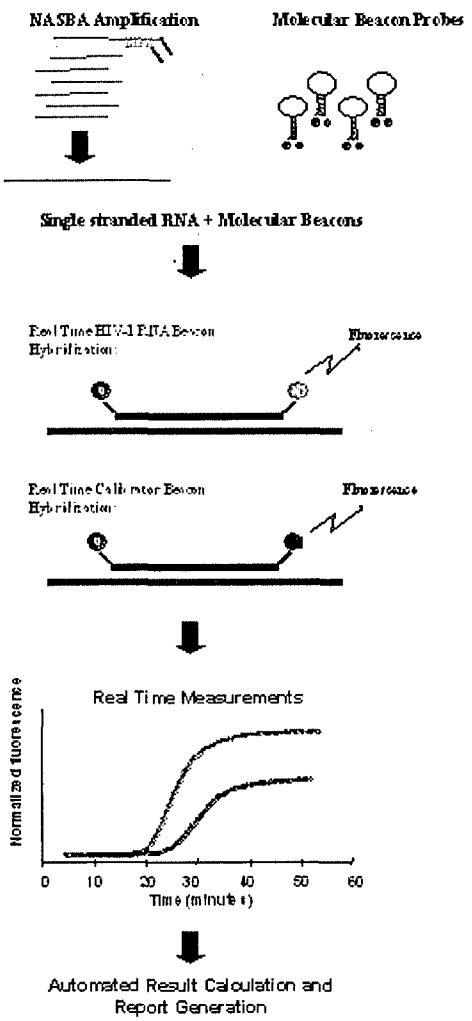

Figure 2. Combination of NASBA with two differently labeled molecular beacon probes in a quantitative test procedure.

In conclusion NucliSens EasyQ features real time amplification and detection in a single, closed tube format. The combination of amplification and detection in a single steps significantly reduces technical hands on time, time to result and cross contamination.

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Tell me more on NucliSens EasyQ

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*The NucliSens Basic Kit does not contain target-specific amplification primers and detection probes, these should be obtained by the user. bioMérieux points out that the use of its NucliSens Basic Kit with specific primers and/or probes may require a license under one or more patent(s) held by third parties. It is the user's responsibility to obtain such license(s). bioMérieux does not support or encourage the unlicensed or unauthorized use of its NucliSens Basic Kit and disclaims any liability resulting from such use.