A Guide for Clinicians in the Evaluation of Emerging Molecular Diagnostics for Newly Diagnosed Prostate Cancer

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Prostate-specific antigen (PSA) screening is associated with a decline in prostate cancer-related mortality. However, screening has also led to overdiagnosis and overtreatment of clinically insignificant tumors. Recently, certain national guidelines (eg, US Preventive Services Task Force) have recommended against PSA screening, which may lead to a reverse-stage migration. Although many prostate tumors are indolent at presentation, others are aggressive and are appropriate targets for treatment interventions. Utilization of molecular markers may improve our ability to measure tumor biology and allow better discrimination of indolent and aggressive tumors at diagnosis. Many emerging commercial molecular diagnostic assays have been designed to provide more accurate risk stratification for newly diagnosed prostate cancer. Unfamiliarity with molecular diagnostics may make it challenging for some clinicians to navigate and interpret the medical literature to ascertain whether particular assays are appropriately developed and validated for clinical use. Herein, the authors provide a framework for practitioners to use when assessing new tissue-based molecular assays. This review outlines aspects of assay development, clinical and analytic validation and clinical utility studies, and regulatory issues, which collectively determine whether tests (1) are actionable for specific clinical indications, (2) measurably influence treatment decisions, and (3) are sufficiently validated to warrant incorporation into clinical practice.

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KEY WORDS

Adverse pathology • Biomarker • Clinical utility • Clinical validation • Genomic prostate score • Molecular diagnostics • Prostate cancer

There is a growing consensus that many prostate cancers identified by prostate-specific antigen (PSA) screening are indolent and could be appropriately managed with active surveillance. However, a proportion of PSA-diagnosed, early-stage prostate cancers are biologically aggressive and should be considered for immediate treatment.[1] The discrimination of these two types of disease is arguably the greatest challenge in the management of newly diagnosed prostate cancer today.

Prediction of outcomes and treatment decisions for prostate cancer are currently based on various nomograms and risk groupings that incorporate clinical stage, serum PSA, and histologic features on diagnostic needle biopsy.[2-4] Although these tools have important prognostic value, numerous series have demonstrated that, in approximately 30% of cases, patients are found to have higher-grade and/or -stage disease than predicted by their biopsy features, highlighting the risk of undersampling inherent to needle biopsies.[5] Conversely, not all patients with biopsies containing Gleason grade 4 disease prove to have aggressive disease at surgery.[6] These discrepancies make it challenging for clinicians to select the appropriate treatment with confidence. Molecular diagnostic assays that provide more accurate measures of tumor aggressiveness may help address this unmet medical need.

Molecular diagnostics can provide an objective biologic measure of tumor aggressiveness. Prostate cancer is a complex and highly variable disease with multiple genomic alterations that influence a wide range of biologic pathways, which collectively determine the aggressiveness of a given tumor. The hallmarks of cancer include (1) sustained proliferation, (2) evasion of growth suppressors, (3) resistance of cell death, (4) enabling of replicative immortality ("immortalization"), (5) induction of angiogenesis, and (6) activation of invasion and metastasis.[7] In addition, tumor tissues are composed of multiple interacting cell types, including malignant cells and tumor-associated stroma, which jointly participate in the process of tumor growth and progression. Multiplex molecular markers can provide a window on the contribution of these various biologic processes to the behavior of individual cancers.

Molecular diagnostics can provide an objective biologic measure of tumor aggressiveness.

A number of tissue-based, multigene expression assays are now clinically available for measuring tumor aggressiveness in prostate cancer. This review provides guidance for clinicians on assessing the validity and utility of these types of genomic tests. The gene expression tests that have the most mature data and are commercially available—the Oncotype DX® Prostate Cancer Test (Genomic Prostate Score [GPS] assay; Genomic Health, Redwood City, CA), which assesses diagnostic biopsy samples at the time of diagnosis; the Decipher® test (GenomeDX Biosciences, San Diego, CA), which assesses post-prostatectomy specimens; and the Prolaris® test (Myriad Genetics, Salt Lake City, UT), which assesses either specimen type—serve as the examples in this review (Table 1).

The key components in assessing any new diagnostic assays for prostate cancer include understanding the following: (1) how the assay was developed, and what genetic pathways and abnormalities are measured by the test; (2) what clinical need is addressed by the assay and what its clinical indications are (is the assay fit-for-purpose?); (3) what type and amount of tissue is required for the assay, and its cost; and (4) what the level of evidence that has been validated is, being mindful of the three key components of assay validation (analytic validation, clinical validation, and clinical utility; Table 2).

Molecular diagnostic assays are new to the clinical care of prostate cancer, and no multiplex genomic assays are currently included in the guidelines for prostate cancer of the National Comprehensive Cancer Network (NCCN) or the American Urological Association. Few multiplex gene expression assays are currently in clinical practice for any solid tumors. One example that has been rigorously validated and incorporated into clinical guidelines for breast cancer[8] is the 21-gene Recurrence Score® assay (Oncotype DX breast cancer test; Genomic Health, Redwood City, CA), which is indicated for use in patients with early breast cancer to inform decisions regarding adjuvant chemotherapy.[9] We refer to this as a "case study" for the development and validation of such a test.

Molecular Diagnostics 101: Molecular Diagnostics Terminology

A biomarker can be broadly defined as any measurable biologic entity whose presence signifies a disease or biologic condition. Molecular diagnostics can include nucleic acids (RNA or DNA), protein, and metabolite biomarkers, which can be measured in a variety of human specimens (eg, tissue, plasma, or urine). Nucleic acid–based assays may include measurement of gene expression in messenger RNA, detection of genetic abnormalities such

TABLE 1

Gene Expression Assays

Company	Assay Name	Indication	Result
Genomic Health Inc. (Redwood City, CA)	Oncotype DX® Prostate Cancer assay	Positive prostate biopsy	Genomic prostate score from 0 to 100 Likelihood of freedom from higher Gleason score[a] and/or non–organ-confined disease
GenomeDX Biosciences (San Diego, CA)	Decipher®	Post radical prostatectomy	Risk of metastasis at 5 years post prostatectomy and 3 years post PSA recurrence
Myriad Genetics (Salt Lake city, UT)	Prolaris®	Positive prostate biopsy or Post radical prostatectomy	10-year PCSM risk or 10-year BCR risk

BCR, biochemical recurrence; PCSM, prostate cancer-specific mortality; PSA, prostate-specific antigen.
[a]High Gleason score defined as Gleason dominant pattern 4 or any Gleason pattern 5.

TABLE 2

Essential Components of Assay Validation

Components of Assay Validation	Definition
Analytic validation	Demonstrating that the assay measures the biomarker(s): it is intended to measure in a robust, accurate, and reproducible manner
Clinical validation	Demonstrating that the assay has a strong association with clinically relevant outcomes (such as adverse pathology at surgery or clinical recurrence)
Clinical utility	Demonstrating that the assay results meaningfully influence the clinical management of the patient and improve net health outcomes

as mutations, gene deletions, and gene fusions, and epigenetic markers such as DNA methylation. The focus of this review is tissue-based, multigene expression assays that measure RNA levels. Commonly used terms are presented in Table 3.

Development of Tissue-Based, Gene Expression Assays

Two major technical challenges for the development of multigene expression assays in prostate cancer bear mentioning: the very small amounts of tissue and RNA available in needle biopsies,[11] and the intrinsic heterogeneity and multifocal nature of most prostate tumors. Given that a standard 12-core prostate needle biopsy samples < 1% of prostate tissue, gene expression patterns observed in the biopsy may not reflect the overall biology of the tumor. The development strategy should optimize assay performance for very small amounts of tumor RNA and mitigate the effects of tumor heterogeneity. A truly powerful biomarker for prostate cancer should be able to predict the same outcomes from any cancer tissue sampled (and perhaps even from adjacent noncancerous tissue). There is exploratory evidence that the Oncotype DX GPS assay and Prolaris test can predict tumor aggressiveness when measured in adjacent tissue that appears normal,[12,13] suggesting that these assays are measuring—in part—a more generalized field effect in the gland and, therefore, may be less susceptible to tumor heterogeneity and multifocality.

The development strategy should optimize assay performance for very small amounts of tumor RNA and mitigate the effects of tumor heterogeneity.

The tests discussed here are all designed to measure the expression of multiple genes, using RNA

TABLE 3

Molecular Diagnostics Terminology

Terminology	Description
Biomarker (also called molecular marker and signature marker)	Biological molecule in blood, other body fluids, or tissues that signifies a normal or abnormal process, including a condition or disease; a biomarker may also be used to assess treatment efficacy
DNA methylation	Attachment of a methyl group to DNA at cytosine bases; DNA methylation is correlated with reduced gene transcription
Epigenetics	The study of heritable changes to gene activity (activation; deactivation) without any alterations in the underlying DNA sequence[a]
Gene expression	Process by which information that is encoded in a gene is used by a cell to direct the assembly of a specific protein molecule
Genetics	Study of genes and heredity
Genomics	Study of the complete genome of an organism, which includes genes and their functions
mtDNA	DNA that is present in cell mitochondria represents a small fraction of the total DNA in cells; mtDNA contains 37 genes; mtDNA is prone to somatic (a type of noninherited) mutations; mtDNA mutations are associated with numerous chronic diseases, including cancer
Phenotype	Observable physical and/or biochemical characteristics of gene expression[a]
Proteomics	The study of the structure, function, and interaction of proteins produced by specific genes. Various techniques (ie, molecular biology, genetics, and biochemistry) may be utilized
RT	Process whereby the RT enzyme enables the transcription of RNA to DNA (retroviruses [eg, HIV], which are RNA viruses, utilize RT to replicate)
Types of laboratory assays	
Microarray-based gene expression profiling	Simultaneous analysis of mRNA expression levels of multiple genes in a tumor specimen; both the Oncotype DX® Prostate Cancer Assay and the Prolaris test utilize a microarray platform[b]
PCR	Qualitative amplification of DNA
qPCR	Amplification and quantitation of DNA
RT-PCR (qualitative)	Following reverse transcription of RNA into cDNA, multiple copies of cDNA are then generated via PCR, enabling *qualitative* detection of gene expression
qRT-PCR (quantitative)	Sensitive assay that enables both detection and *quantitation* of gene expression by creating multiple copies of cDNA from RNA, followed by cDNA amplification with specialized probes

[a]Data from National Human Genome Research Institute Web site.[10]
[b]The Oncotype Dx® Prostate Cancer Assay is manufactured by Genomic Health (Redwood City, CA); the Prolaris test is manufactured by Myriad Genetics (Salt Lake city, UT).
cDNA, complementary DNA; mtDNA, mitochondrial DNA; PCR, polymerase chain reaction; qPCR, quantitative PCR; qRT-PCR, quantitation real-time PCR; RT, reverse transcription.

extracted from formalin-fixed tumor tissue, and to provide a biologic measure of tumor aggressiveness. However, assay development and gene selection strategies for each test were different.

Given the contribution of cell proliferation to tumor aggressiveness, the Prolaris test was developed by a narrowly focused screening of 126 cell cycle genes and was not intended to measure other biologic pathways, such as tumor invasiveness. Genes were selected based on their correlation with the mean expression of all 126 genes. The resulting commercial assay, which can be performed on needle biopsy material and on radical prostatectomy tissue, consists of 31 highly correlated cell cycle progression (CCP) genes and 15 housekeeping genes (to correct for variable RNA quality and quantity), and provides

a CCP score that ranges from −2 to +6, with higher numbers reflecting increased proliferation.[14]

The Oncotype DX GPS test was developed based on a screening of 732 cancer-related genes, representing a wide range of biologic pathways, to determine which genes were most predictive of clinical recurrence after surgery.[15] Genes were selected based on their ability to predict clinical recurrence, biochemical recurrence, prostate cancer-specific mortality, and adverse pathology, when assessed in two different regions of a given tumor, to mitigate the confounding effects of tumor heterogeneity, and then further selected by their analytic performance in biopsy tissue. The final biopsy-based assay consists of 12 cancer-related genes (representing androgen signaling, stromal response, cellular organization, and proliferation) and 5 reference genes, and provides a GPS on a 100-unit scale. It is currently not intended for use on radical prostatectomy specimens.

The genes in the Decipher assay were selected based on a screening of more than 1.4 million probes using a GeneChip® Human Exon ST Array (Affymetrix, Santa Clara, CA).[16] A 22-gene genomic classifier was developed, using a random forest machine learning algorithm, as a predictor of clinical recurrence after surgery. The 22 genes selected for the final assay, which is performed on radical prostatectomy tissue, reflect a variety of biologic pathways, such as cell proliferation, differentiation, and motility, and include noncoding RNAs.

Analytic Validation

For a test to be analytically validated, it must be proven robust and reliable (eg, in the face of various fixatives, variable amounts of input nucleic acid), and measures the analyte it claims to measure. Analytic validation studies determine the test accuracy, precision, and reproducibility (ie, if the results are consistent between duplicate samples, different operators, different instruments, different reagent lots, and over different time periods), and includes the determination of quality control measures for the assay.[17] Standards for the conduct and reporting of analytic validation studies have been recently reviewed.[18] The analytic validation studies for the Oncotype DX GPS test demonstrated that all of the genes in the test met prespecified criteria for precision and reproducibility.[11]

Clinical Validation

For an assay to be clinically validated, a high level of evidence must show that the biomarkers that the test measures have a strong association with one or more clinically relevant outcomes. In prostate cancer, this can include the prediction of adverse pathology at surgery, biochemical recurrence, development of metastases, and prostate cancer death.

In 1996, a grading system was proposed to define levels of evidence for clinical validation of tumor markers.[19] The traditional gold standard for a clinical validation study was a prospective randomized trial designed specifically to determine if an assay was a significant predictor of a clinically relevant endpoint.[17] However, given the extended natural history of prostate cancer, prospective trials with long-term clinical endpoints are not practical. In 2009, the authors modified their algorithm, which assigns levels of evidence (from I to IV), to indicate that retrospective studies of archival tissues can provide Level 1 evidence of clinical validity (Table 4),[20] if they are rigorously designed and conducted; have prestated eligibility criteria, specific aims, and statistical analysis plans; and are performed on patient cohorts that are representative of the contemporary target population for the assay. Lastly, for a study to be considered a true clinical validation study, it should be performed using the final, analytically validated commercial-grade version of the biomarker assay.

It should be noted that even retrospective studies that assess late endpoints are challenging to conduct, as there are few mature cohorts with available archival tissue, and randomized trials in early prostate cancer are extremely uncommon. Patients in many of these older cohorts were not screened or managed in accordance with contemporary practices. Thus, more near-term endpoints, such as biochemical recurrence and adverse pathology (high-grade and/or high-stage disease) at surgery, are more feasible as surrogates for later clinical events.

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The three assays discussed here can be compared with these standards. The Oncotype DX GPS has been clinically validated, in two independent cohorts, to predict the likelihood of high-grade and/or non–organ-confined disease at radical prostatectomy ($P < .005$) in men with NCCN low and intermediate disease when performed on pretreatment diagnostic needle biopsy specimens.[21] The assay has

TABLE 4

Determination of LOE Using Elements of Tumor Marker Studies

LOE	LOE Category	Study Design	Validation Studies Available
I	A	Prospective	None required
	B	Prospective using archive samples	≥ 1 with consistent results
II	B	Prospective using archive samples	None or inconsistent results
	C	Prospective/observational	≥ 2 with consistent results
III	C	Prospective/observational	None or 1 with consistent results or inconsistent results
IV-V	D	Prospective/observational	Not applicable[a]

[a]Not applicable because LOE IV and V studies will never be satisfactory for the determination of medical utility.
LOE, level of evidence.
Reproduced from Simon RM, Paik S, Hayes DF. Use of archived specimens in evaluation of prognostic and predictive biomarkers. *J Natl Cancer Inst.* 2009;101: 1446-1452, by permission of Oxford University Press.[20]

also been validated as a significant independent predictor of biochemical recurrence after surgery for localized prostate cancer.[22] The Prolaris CCP score has been clinically validated to predict biochemical recurrence after surgery when performed on radical prostatectomy specimens and to predict prostate cancer-specific death when performed on transurethral resection of the prostate[14] or diagnostic needle biopsies.[23] The Decipher assay has been clinically validated to predict the likelihood of developing metastatic disease after radical prostatectomy when performed on surgical tissue.[16] Thus, all three assays can be considered to have Level 1B evidence for clinical validation (ie, ≥ 1 prospectively designed study of archival tissues [Table 4]).

Clinical Utility

Does the assay affect patient outcomes and treatment decisions in a meaningful way? An ideal clinical utility study for a cancer assay would demonstrate that use of a particular test is associated with a reduction in cancer-related mortality. The long natural history of prostate cancer makes such studies impractical. Subsequently, most clinical utility studies focus on short-term endpoints, such as changes in the treatment decisions that are likely to improve long-term outcomes. For example, does using the assay drive greater adoption of active surveillance in good candidates, thereby reducing harms associated with overtreatment, without having a negative effect on overall outcome?

Such "decision impact" studies for prostate cancer tests have been conducted a number of different ways. Badani and colleagues[24] studied the clinical utility of the Decipher test in the postprostatectomy setting with online surveys using hypothetical cases, for which physicians were asked to indicate their treatment recommendations before and after receiving the result of the test. They observed a 43% to 53% post-test change in treatment recommendations regarding adjuvant or salvage therapy. Crawford and colleagues[25] assessed the clinical utility of the Prolaris test in a prospective study of newly diagnosed patients, in which their urologists completed questionnaires indicating their treatment recommendations prior to and after receiving the test results. They observed a 65% change in post-test treatment recommendations, with a 37% reduction in recommendations for interventional therapies. Kartha and associates[26] conducted a single-institution study of the clinical utility of the Oncotype DX GPS test on risk stratification and treatment decisions for 115 men with newly diagnosed prostate cancer. They observed a 21% change in risk stratification in the total cohort, and a 46% change (44% to very low risk) in men with NCCN low-risk disease. The authors noted that physician treatment recommendations changed in every case in which the risk stratification was altered by the test result and that patients accepted the physician recommendation in all but one case.

The results of physician questionnaire–based studies must be interpreted with caution, as they may not reflect real-life clinical practice and may be subject to the Hawthorne effect (also referred to as "observer effect"). The Hawthorne effect is described as subjects' awareness of their participation in an experiment, which can affect their behavior and, ultimately, the results of an experiment.[27] In addition, it is unclear if the post-test recommendation is actually carried out. Strategies to ensure that such

studies reflect real-life clinical decision making include (1) capturing physician-reported levels of decisional confidence and usefulness of the test, (2) including patient preferences and patient-reported measures of decision conflict, and (3) systematic post-test chart reviews to document the actual management course taken in comparison with matched controls of untested patients. To ensure that a test has broad clinical utility, these studies should represent a range of practice types, patient demographics, and geographic locations.

Regulatory Issues: What Regulatory Approvals Are Required for Molecular Diagnostics?

Although molecular diagnostic tests in the United States are regulated by the US Food and Drug Administration (FDA) and the Centers for Medicare & Medicaid Services (CMS), the FDA has exercised discretion in its requirements for clearance and approval of in vitro diagnostic assays, limiting them to distributed tests (tests sold as kits to commercial laboratories) and companion diagnostics required to select patients for specific drug treatments.[28,29] Laboratory-developed assays (in vitro tests developed, validated, and performed in-house by a specific reference laboratory) are required to abide by the Clinical Laboratory Improvement Amendments (CLIA) of 1988, which are administered by CMS. CLIA aims to ensure reliability and accuracy of test results and establish quality standards for all laboratory testing. The three prostate cancer tests under consideration here all fall under the CLIA umbrella. Whereas FDA clearance requires demonstration of clinical and analytical validation but not clinical utility, all three components are typically required for inclusion in clinical cancer guidelines and for payer reimbursement.

A Case Study of a Fully Validated Molecular Diagnostic for Breast Cancer

The 21-gene Recurrence Score assay (Genomic Health, Inc., Redwood City, CA) is an example of a mature molecular diagnostics test that has satisfied all three measures of assay validation—analytic validation, clinical validation, and clinical utility—and is used to help guide treatment decisions regarding adjuvant chemotherapy for women with localized breast cancer. As a consequence of its comprehensive validation, the test is included by various cancer guidelines, including those of the American Society of Clinical Oncology, NCCN, and National Institute for Health and Care Excellence.[2]

Although molecular diagnostic tests in the United States are regulated by the FDA and CMS, the FDA has exercised discretion in its requirements for clearance and approval of in vitro diagnostic assays, limiting them to distributed tests and companion diagnostics required to select patients for specific drug treatments.

The analytic validation studies for the assay demonstrated that the amplification efficiency, precision, linearity, and dynamic range for each gene in the test met prespecified criteria for analytic performance, and the test was reproducible as described earlier[30] and has been cited as a model for the conduct and reporting of analytic validation studies.[18]

The Recurrence Score assay has been clinically validated in women with early breast cancer to predict the likelihood of (1) 10-year distant recurrence in estrogen receptor–positive, tamoxifen-treated patients[31] (2) benefit from adjuvant chemotherapy,[32] and (3) breast cancer-related death in the absence of adjuvant chemotherapy.[33] In a 2012 analysis of molecular assays for predicting outcomes in patients with early-stage breast cancer, category I level of evidence was achieved only in studies validating this assay.[34]

For clinical utility, the use of the Recurrence Score has been shown to alter treatment recommendations in 30% to 40% of patients in several decision impact studies.[35] In most cases, the shift was from adjuvant chemotherapy/endocrine therapy to endocrine therapy alone, sparing a substantial fraction of women the toxicity and expense of chemotherapy. These studies also demonstrated that physician confidence in their treatment recommendations improved by 45% ($P < .001$), and patient decisional conflict was reduced by the Recurrence Score results.

Additional Considerations and Conclusions

Two major technical challenges for the development of multigene expression assays in prostate cancer deserve some mention. They are the very small amounts of tissue and RNA available in needle biopsies,[11] and the intrinsic heterogeneity and multifocal nature of most prostate tumors. Given that a standard 12-core prostate needle biopsy samples < 1% of prostate tissue, gene expression patterns observed in the biopsy may not reflect the overall biology of the tumor. The development strategy should optimize assay performance for very small amounts of tumor RNA, and

mitigate effects of tumor heterogeneity. A truly powerful biomarker for prostate cancer should be able to predict the same outcomes from any cancer tissue sampled (and perhaps even from adjacent noncancerous tissue). There is exploratory evidence that the Oncotype DX GPS assay and Prolaris test can predict tumor aggressiveness when measured in adjacent normal-appearing tissue,[12,13] suggesting that these assays are measuring—in part—a more generalized field effect in the gland and may, therefore, be less susceptible to tumor heterogeneity and multifocality.

The key attributes of a clinical biomarker are that (1) it has a strong association with clinically meaningful endpoints, be they pathologic or clinical, (2) it provides additional information about tumor biology beyond existing clinical nomograms, and (3) use of the marker has a meaningful influence on physicians' treatment decisions and patient outcomes. In the end, the assay should permit more accurate risk assessment for patients with early-stage prostate cancer and more individualized treatment decisions. ■

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MAIN POINTS

- Many prostate cancers identified by prostate-specific antigen (PSA) screening are indolent and could be appropriately managed with active surveillance. However, some PSA-diagnosed early-stage prostate cancers are biologically aggressive and should be treated immediately. The discrimination of indolent from aggressive of disease is arguably the greatest challenge in the management of newly diagnosed prostate cancer today; molecular diagnostic assays that provide more accurate measures of tumor aggressiveness may help address this unmet medical need.
- Assay development, clinical and analytic validation, and clinical utility studies collectively determine whether tests are actionable for specific clinical indications, measurably influence treatment decisions, and are sufficiently validated to warrant incorporation into clinical practice.
- A number of tissue-based, multigene expression assays are now clinically available for measuring tumor aggressiveness in prostate cancer. These include the Oncotype DX® Prostate Cancer Test (Genomic Prostate Score assay; Genomic Health, Redwood City, CA); the Decipher® test (GenomeDX Biosciences, San Diego, CA); and the Prolaris® test (Myriad Genetics, Salt Lake City, UT).
- Each of these tests is designed to measure the expression of multiple genes using RNA extracted from formalin-fixed tumor tissue, and to provide a biologic measure of tumor aggressiveness.
- An assay should permit more accurate risk assessment for patients with early-stage prostate cancer and more individualized treatment decisions.

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