Systems Pathology

A Paradigm Shift in the Practice of Diagnostic and Predictive Pathology*

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Diagnostic tumor pathology in the context of personalized medicine has progressed from an interpretive, subjective science to a more objective, evidence-based practice. This has resulted in the development of several tissue-based, molecular-driven tests that provide information regarding prognosis and response to therapy. The challenge, however, for both the pathologist and the treating physician is how best to effectively integrate this data into a comprehensive treatment plan that includes a patient-specific risk assessment. To address this need, the authors developed a systems pathology approach to the practice of clinical molecular medicine through technical advances in object-oriented image analysis, and phenotyping at the microanatomical level using deparaffinized tissue section and quantitative biomarker multiplexing. With support vector regression for censored data, they have been able to integrate complex information and provide a patient-specific risk profile based on the clinical endpoint under investigation. **Cancer 2009;115(13 suppl):3078–84. © 2009 American Cancer Society.**

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Systems pathology represents a major advance in the standard practice of tissue-based pathology through its integration of molecular and imaging data with the patients' clinical history.[1,2] These dissimilar data sets are effectively analyzed with machine learning analytics, which selects features based on their association with a clinical event. This results in a highly accurate multivariate predictive model that identifies an individual's probability of experiencing a specific outcome over time. Our working hypothesis was that by using this approach, and expanding the clinical pathological variables by including standardized and objective morphometric features and molecular biomarkers, we could develop a more robust new tool for predicting patient outcome.

The reduction of a systems-oriented approach to medical practice, however, requires advances in quantitative technical assay development and supervised mathematical tools for data integration.[3] Once established, it is necessary to ensure data reproducibility and dissemination to the clinical community, where issues of efficacy and implementation are critical.[4] The prototype systems pathology format has

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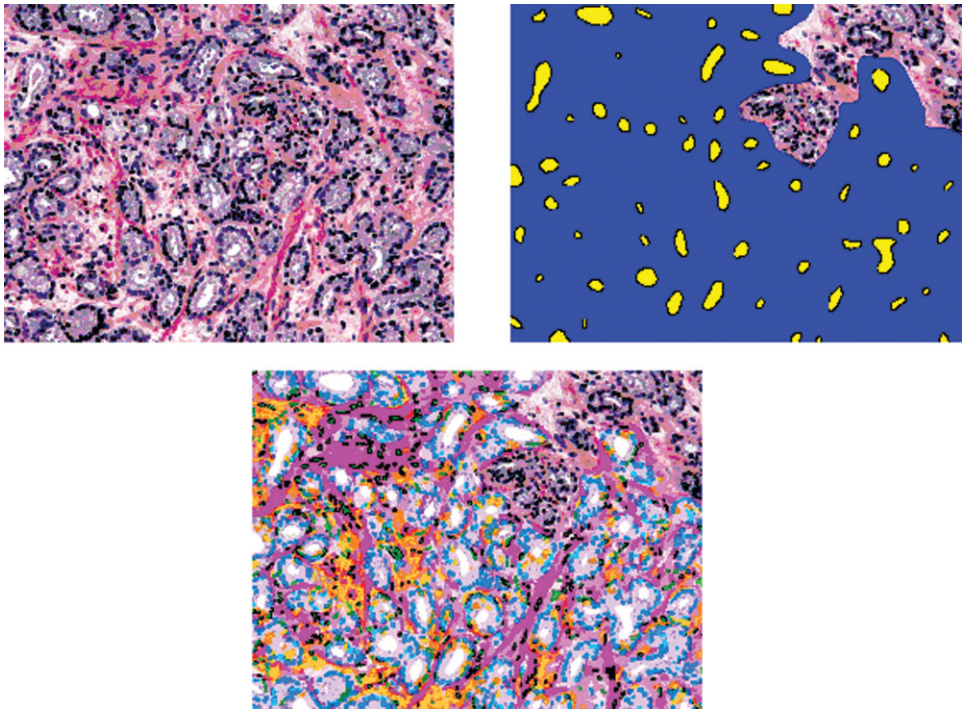

FIGURE 1. A digitized hematoxylin and eosin (H&E)-stained image shows a prostate needle biopsy processed with the histology labeling tool software. A region of infiltrating tumor is identified in the H&E image (*Top left*). The area of invasive tumor has been digitally masked (blue overlay) and tumor gland lumens outlined (yellow; *Top right*). Only the masked field (*Top right*) is processed with the software to produce a segmented and classified image that represents tumor epithelial nuclei (blue), tumor cytoplasm (light purple), stroma (dark and light pink), gland lumens (white), and artifactual spaces (gold-yellow) (original magnification, ×200).

been standardized in a clinical testing environment (eg, CLIA/CLEP/CAP certified) at Aureon Laboratories. Here efforts are underway to provide disease-specific adjunctive tools that have broad applicability in patient management, including the therapeutic decision process. One of the major practical advantages is that objective, reliable parameters are used to accurately risk-stratify patients with respect to their outcome. We believe that an analytical approach will be useful in balancing various risk-based treatment options, particularly evident in the management of patients diagnosed with prostate cancer, this being the proof-of-concept paradigm.

Because the majority of surgical pathology specimens are stored as formalin-fixed, paraffin-embedded (FFPE) materials, we developed assays and an analysis platform that extract quantitative phenotypic data from FFPE sections in a reliable, standardized approach. A significant technical advance was the ability to maximize the amount of information that can be retrieved from an intact tissue specimen, regardless of acquisition method or format (eg, whole section, needle biopsy, fine needle aspirate, tissue microarray). Using pathologist expert knowledge, and prostate cancer as the prototype disease, we developed a custom-made, adaptable image analysis program for hematoxylin and eosin (H&E)-stained tissue sections (Fig. 1).[5-7] In the prostate morphometric analysis, areas of infiltrative tumor and gland lumens are outlined by the pathologist using digital masking software (Fig. 1, *Top right*). The derived features represent characteristics of the general tissue architecture, tumor cell distribution, and composition. In addition, we developed a multiplex, immunofluorescent (IF) spectral approach for measuring biomarkers in FFPE sections using fluorescent tagged antibodies. (Fig. 2A and B).[6,7] The ability to assess a given biomarker on a continuous scale as opposed to more traditional subjective and nominal methods (ie, staining index) has allowed us to more accurately stratify patients with respect to their individual risk. In the assay, a series of

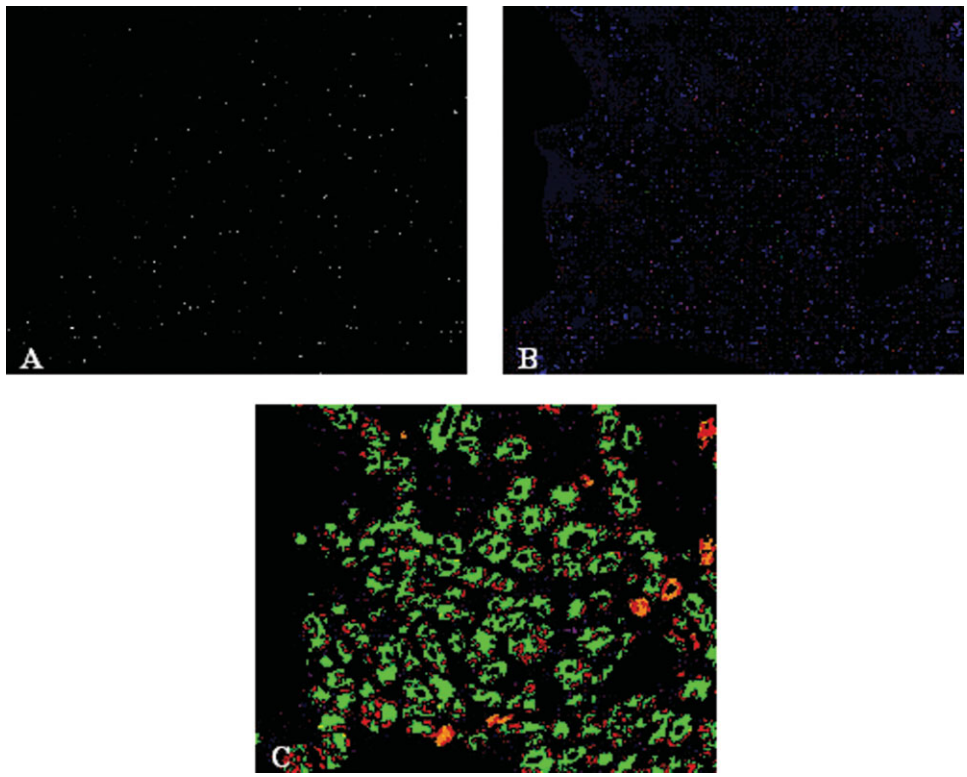

FIGURE 2. Using multiplex immunofluorescence, a series of protein biomarkers including cytokeratin 18, alpha-methyl CoA racemase (AMACR), and the androgen receptor (AR) are evaluated in formalin-fixed, paraffin-embedded prostate needle biopsy tissue sections. Nuclear AR profiles (white dots) are first examined as gray scale images (A). The gray scale views are processed using a layering algorithm that assigns 4′-6-diamidino-2-phenylindole–positive nuclei as blue and nuclear AR as pink-red. After segmentation and classification, compact gland units are seen as green (CKI8+/AMACR−) or orange (CKI8+/AMACR+), and variation in AR intensity is illustrated as red (intermediate) to yellow (high) based on threshold parameters within the script.

antibodies are differentially labeled with Alexa fluorochromes and incorporated into a multiplex format used on FFPE sections. The IF images are acquired with a CRI spectral imaging system (Woburn, Mass), unmixed, and then digitally masked to remove artifacts, autofluorescence, and benign structures. Images are processed with proprietary software to generate a segmented and classified cell and tissue-specific object-oriented image (Fig 2C). For each biomarker, an intensity threshold strategy is applied that incorporates pathologist-assisted, mathematical models to determine optimal expression levels as a function of image background characteristics. Normalization methods have been developed to adjust for variable tumor content between patient samples. The H&E and IF analyses generate several hundred tumor-specific features that are then evaluated using a univariate concordance index with respect to outcome and their association with known, perceived biologic function. Appropriate standards, including prostate cancer cell lines (eg, LNCaP, DU145, and PC3) and control prostate tissue samples, are routinely used to ensure standardization of assay performance.

Application of Systems Pathology to Prostate Cancer Prediction

The systems pathology method has been successfully applied to predict the likelihood of disease recurrence using prostatectomy materials in a tissue microarray. Patient's diagnosed with prostate cancer are confronted with a confusing decision process that has been exacerbated by the lack of consistency and standardization currently used by leading urologic organizations.[8] Even after a treatment, deciding on the appropriate surveillance

strategy and/or management of a disease recurrence has not been straightforward. Postprostatectomy nomograms have provided reasonable accuracy metrics with respect to outcome; however, for the majority of patients with mid-range clinical features and probabilities, these models offer little more assurance than a coin toss.[9-12] Our initial investigative studies focused on predicting disease recurrence after a curative intent radical prostatectomy primarily because of accessible patient tissue samples and available continuous follow-up. The combined cohort of 758 patients used to develop the models was randomized and evenly split between a training and validation set with balanced demographics and clinical outcome events. All patients had been treated with radical prostatectomy between 1985 and 2003 for localized and locally advanced prostate cancer. We excluded patients who received treatment either before prostatectomy or immediately after but before biochemical recurrence.

Two initial models were developed that predict: 1) the likelihood of prostate-specific antigen (PSA) recurrence and 2) clinical failure (eg, castrate PSA rise, systemic metastasis, and/or death from prostate cancer) 5 years after a radical prostatectomy.[6,7] Both the PSA recurrence and clinical failure (CF) predictive models are independently generated and integrate the biomarker data with the patient's clinical and imaging features, therefore predicting levels of risk for a defined clinical outcome. Both prognostic models are currently available to patients and urologists as part of the Prostate Px test provided by Aureon Laboratories, Inc, Yonkers, New York (www.aureon.com).

Predictive accuracy was assessed using the concordance index (c-index, similar to the area under the receiver operating characteristic curve) and hazard ratio.[12] A c-index of 0.5 would indicate that the model performs the same as a coin toss, whereas 1.0 would mean that the model has a perfect ability to discriminate. The PSA recurrence model had a c-index of 0.77, and a hazard ratio of 5.5 ($P < .0001$), whereas the systemic metastasis model had a c-index of 0.84 and hazard ration of 11.4 ($P < .0001$). Both models provide correlative prognostic information, which we believe should be part of the treatment algorithm after a radical prostatectomy. By way of comparison, when the Kattan 5-year postoperative PSA recurrence nomogram was applied to the validation cohort of the CF model, the resulting c-index was 0.75 versus 0.84 for the systems model.[7,8] Such predictive accuracy measures are important when trying to resolve several competing risk-based patient management decisions.

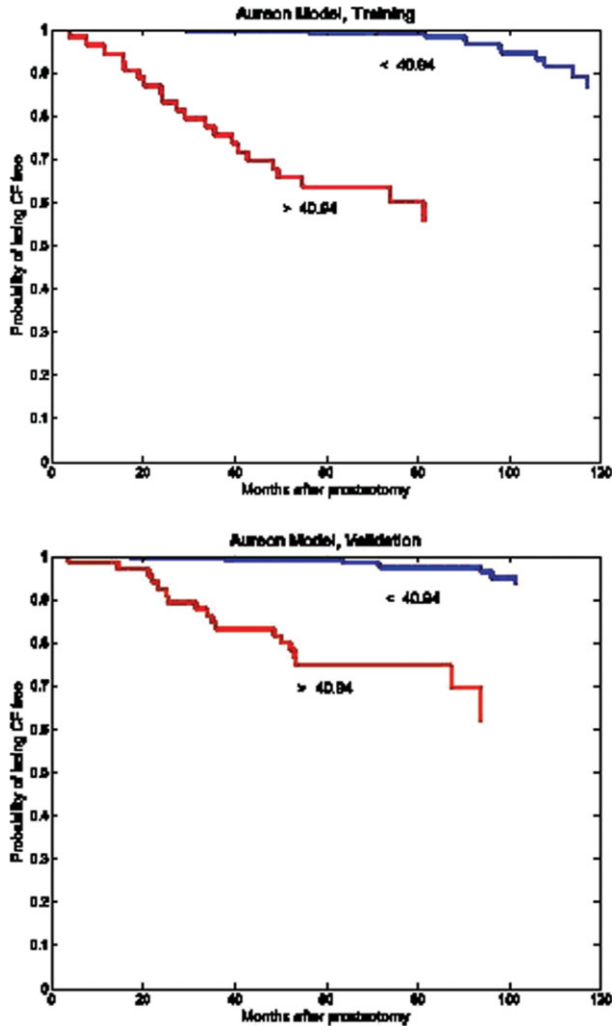

FIGURE 3. Kaplan-Meier curves illustrate stratification of patients based on progression of disease from the clinical failure (CF) training (*Top*) and validation (*Bottom*) model. Patients were stratified into high (red line) and low (blue line) risk groups based on their support vector regression for censored data model score (high risk, >40.94; low-risk, ≤40.94). The probability of remaining clinical failure free is provided by the y-axis, and the follow-up time (in months) is given by the x-axis. The *P* value (<.0001) is estimated using the log-rank test.

The prostatectomy tissue specimens were evaluated using H&E histomorphometry and quantitative biomarker immunofluorescence assays to generate tumor-specific features. For the multiplex IF prostatectomy assay, we used 4′-6-diamidino-2-phenylindole (DAPI) to

Table 1. Selected Features in the PSA Recurrence and Clinical Failure Models, Listed in Decreasing Order of Importance (Weight) Within the Respective Model

PSA Recurrence Feature Set	Clinical Failure Feature Set
Biopsy Gleason sum	Lymph node invasion
Seminal vesicle invasion	Variation in staining properties of tumor cytoplasm by H&E
Variation in the staining properties of tumor epithelial nuclei by H&E	Border length of gland lumens
Variation in the staining properties of tumor epithelial cytoplasm by H&E	Dominant prostatectomy Gleason grade
PSA	Relative area of gland lumens
Dominant prostatectomy Gleason grade	Average intensity of AR in AMACR(−) tumor cells
Relative area of AR in tumor epithelial nuclei	
Extracapsular extension	

PSA indicates prostate-specific antigen; H&E, hematoxylin and eosin; AR, androgen receptor; AMACR, alpha-methyl-CoA racemase.

label nuclei, and then assayed 5 antibodies including cytokeratin 18 (CK18), high–molecular-weight keratin, p63, alpha-methyl-CoA racemase (AMACR), and androgen receptor (AR). On the basis of the DAPI and CK18 staining, the histologic labeling tool software delineated specific tissue features (eg, stroma vs glandular epithelium). Appropriate algorithms were then formulated for quantifying AR within these regions. The algorithms measure the mean, median, maximum, and standard deviation of AR (protein) intensity and distribution in epithelial and stromal nuclei. As part of the quality assurance procedure, control prostate cancer tissues and 3 prostate cancer cell lines (eg, LNCaP, DU145, and PC3) were routinely evaluated with the same software used to derive the IF features for inclusion in the model.

The Kaplan-Meier curves for training and validation of the clinical failure model illustrate the ability to stratify patients into low- and high-risk categories based on their individual support vector regression for censored data model scores (Fig. 3). Because the models are time-dependent, the selected features reflect both the predicted clinical endpoint and underlying mechanism of disease (Table 1). Of importance, both the PSA recurrence and clinical failure models showed that increased expression of the AR was associated with disease progression. Furthermore, patients who had high levels of AR in the CF study were resistant to standard androgen ablation therapies. This would suggest that patients with elevated AR are at increased risk for progression of their disease, and that standard therapies may not be appropriate and/or sufficient. There is good evidence in the literature that signaling via AR is an important contributor to disease progression.[13-15] Given that AR is present in benign as well as prostate tumor cells, the ability to discriminate AR levels associated with progression of disease has been challenging. Li et al[15] and Inoue et al[16] both demonstrated that high levels of AR were associated with treatment failure; however, these analyses were performed with a semiquantitative immunohistochemical assay prone to subjective interpretation and difficult to standardize. The data from the systems pathology models, using quantitative assays that provide reproducibility and feature robustness, have suggested that providing AR status in the prostatectomy specimen would be potentially beneficial in the development of a specific therapeutic plan (eg, salvage radiation ± androgen therapy).

We recognized early on that although the postprostatectomy models supplied useful information, there was a defined clinical need to provide measures of risk at the time of diagnosis using the prostate needle biopsy and pretreatment clinical variables. In the current PSA screening era, more men are being diagnosed with localized prostate cancer. Unfortunately, how to identify the patient who is at high risk for clinically significant disease remains unclear.[17,18] Using prostate needle biopsy specimens from men with T1c-T3 stage prostate cancer, who had been treated by curative-intent radical prostatectomy and followed for 8 years, we have developed a test that offers in each case the likelihood of disease progression (castrate PSA rise and/or systemic metastasis). Briefly, the study demonstrates that a model trained on 686 patients can successfully predict progression of disease with 74% predictive accuracy, and a hazard ratio of 5.12. By focusing our efforts on a cohort with a high percentage of intermediate-risk patient all treated with curative-intent surgery, we optimized our ability to accurately discriminate

patients with indolent versus aggressive disease. We have validated the approach using an independent cohort of 341 patients, and achieved a predictive accuracy of 73%, with a hazard ratio of 3.47. Furthermore, our model reveals the importance of quantitative histology and biomarker features (eg, AR and Ki67) in the discrimination of disease progression risk among intermediate-grade prostate cancers. By comparison, the Kattan 5-year biochemical preoperative recurrence nomogram,[19] when applied to the same cohort, yielded a predictive accuracy of 69% and a hazard ratio of 2.34, demonstrating the improved accuracy with a more clinically relevant endpoint, obtained with this novel approach. In sum, we believe that the present model, which employs multiple robust tumor characteristics and biomarkers, yields a more objective risk assessment of contemporary patients, particularly in a community practice, where selected pathologic variables are prone to subjectivity.

In addition, using slightly different yet overlapping features, the test also provides a probability for whether the patient will have organ-confined, good-outcome pathologic stage "favorable pathology" (eg, prostatectomy Gleason grade of $\leq$6, $\leq$pT2, and clinical evidence of PSA nadir). As with the disease progression model, advanced histomorphometric strategies and additional biomarkers were included to isolate subtle differences between Gleason grade 3 and 4 patterns in biopsy tissue specimens. This is critical, as the most challenging aspect of patient management is those with intermediate risk factors such as Gleason score of 7 and PSA levels between 10 and 20 ng/mL. We believe that such efforts will objectively assess the risk stratification process and identify biologic correlates useful for understanding disease pathways and possibly guiding selective therapies.

Summary

Systems pathology represents a methodological shift in how traditional diagnostic pathology is currently performed using deparaffinized tissue sections. The technical advances outlined above exemplify ways in which phenotypic characteristics contained within the diagnostic specimen can be routinely extracted and used, in a clinical setting, for routine patient management. We believe that the incorporation of a systems-based approach through functional histology will provide a framework for further advancing personalized medicine and selective therapy design.

Conflict of Interest Disclosures

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