Clinical Validation of a live-cell phenotypic biomarker - based diagnostic assay for the prediction of adverse pathology in Prostate Cancer

David Albala MD[1], Vladimir Mouraviev MD[2], Kimberly Rieger−Christ PhD[3], Travis Sullivan MS[3], Naveen Kella MD[4], Kevin Knopf MD[5], Hani Rashid MD[6], Michael Manak PhD[7], Brad Hogan PhD[7], Gauri Dixit PhD[7], Delaney Berger BA[7], Wendell Su MS[7], Matthew Whitfield PhD[7], Jonathan Varsanik PhD[7], Mani Foroohar MD[7], Stephen Zappala MD[8], Ashok C. Chander PhD[7], Grannum R. Sant MD[9] [1]Associated Medical Professionals of New York, Syracuse, NY, [2]Florida Hospital, Orlando, FL, [3]Lahey Hospital and Medical Center, Burlington, MA, [4] The Urology Place, San Antonio, TX, [5]California Medical Center Pacific, San Francisco, CA, [6]University of Rochester Medical Center School of Medicine and Dentistry, Rochester, NY, [7]Cellanyx Diagnostics, Beverly MA, [8]Andover Urology, Andover, MA, [9]Tufts University School of Medicine, Boston, MA.

Introduction and Overall Goal: Prostate cancer accounts for over 28% of total cancer cases in the United States. Current screening and diagnostic approaches lack the sensitivity to objectively assess the aggressiveness of tumors. To address this issue, a diagnostic assay was developed to differentiate indolent from aggressive tumors, objectively risk stratify patients and predict adverse pathology. Here we describe a diagnostic platform that is based on the measurement of a panel of phenotypic and molecular biomarkers in live biopsy-derived cells. Combining microfluidics, automated imaging and image analysis, the assay provides predictive scores for local aggressiveness, invasiveness, and the presence of adverse clinical pathologies.

Specific Aims: (1) Establish conditions to stably maintain live primary prostate cells for automated live-cell and fixed cell imaging and collection of data on phenotypic biomarkers. (2) Measure both cellular and molecular phenotypic biomarkers on a blinded 237 sample set in an automated high-through put manner. (3) Statistically analyze the 237 sample set in an unbiased objective way using automated algorithmic analysis and test the performance of the algorithmic analysis by comparative analysis of the unblinded data.

Rationale: Develop algorithms that can predict adverse pathologies from a sample using either tumor derived cells or cells from adjacent normal tissue in order to capture the tumor behavior or 'field effect' the tumor has on cells from surrounding tissue not directly involved as tumor cells.

Methods: This clinical validation study was done on fresh prostate cancer samples (n=237) obtained at the time of radical prostatectomy in which tumor tissue and adjacent normal tissue (n=67) were procured. Patient cells were grown *ex vivo* (up to 72 hrs) to enable live-cell, label-free imaging of multiple phenotypic biomarkers. Cells were then stained & imaged for molecular markers. Data were objectively quantified by machine vision algorithms to evaluate cellular behavior, and machine learning analysis to generate predictive metrics.

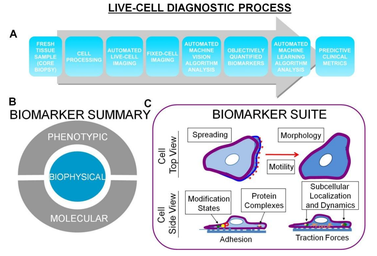
Results: The developed predictive dynamic biomarker metrics of adverse pathology: "Local Adverse Pathology Potential (LAPP)," "Metastatic Adverse Pathology Potential (MAPP)," and "General Adverse Pathology Potential (GAPP)" report on the local aggressiveness, invasiveness, and general likelihood of adverse pathologies, respectively, are able to distinguish benign from malignant cells, risk stratify fresh tumor or adjacent normal samples, and predict adverse pathology. Comparing our results with known clinical pathology data, we can distinguish Gleason 6 from Gleason 7 and Gleason (3+4) from Gleason (4+3) with greater than 85% sensitivity and specificity. LAPP, MAPP, and GAPP metrics can also predict the likelihood of six different adverse clinical pathologies with high accuracy as characterized by Receiver Operator Curves with Area Under the Curve (AUC) values >0.80.

Discussion and Conclusions: This novel live-cell phenotypic assay can quantitatively risk stratify patients with similar Gleason scores. Moreover this first-in-class diagnostic can predict adverse clinical pathologies from either tumor sample directly or adjacent normal tissue, namely 1) seminal vesicle invasion, 2) positive surgical margins, 3) extra prostatic extension, 4) perineural invasion, 5) vascular invasion and 6) lymph node invasion. The algorithms specifically designed to interrogate normal tissue adjacent to tumors resulted in GAPP metrics that give a reading about a patients adverse pathologies even when tumor sample is not directly collected producing a measure of the tumor 'field effect' on surrounding cells. These results suggest that this novel assay can accurately stratify low & intermediate risk cases and aid clinical decision-making to improve treatment outcomes.

INTRODUCTION:

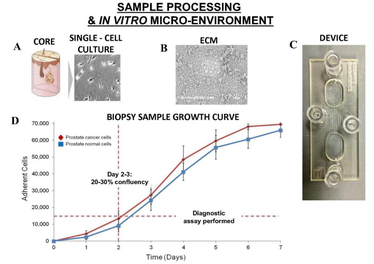
- **Solid tumor cancers such as prostate, cancers are generally diagnosed through a combination of blood tests and macroscopic imaging including x-ray, computer tomography (CT), magnetic resonance imaging (MRI), and ultrasound. These tools do not provide a clear cellular diagnostic or prognostic analysis of the disease.**
- **Only when biopsy samples are imaged, is a microscopic cellular analysis possible. However, the current state-of-the-art does not consider the underlying biology of live biopsy cells, leaving an unclear diagnostic analysis.**
- **Due to the lack of clear diagnostic tools, cancer of the prostate is over diagnosed and over treated (1-3).**
- **There is an urgent need for quantifiable & actionable risk-stratification biomarkers for prostate cancer.**

LIVE-CELL DIAGNOSTIC PROCESS

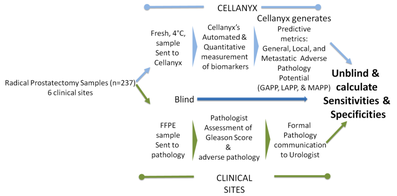


Novel diagnostic platform measures phenotypic (biophysical and molecular) biomarkers on live cells harvested from patient tumor samples. A) Flow diagram outlining the diagnostic process of fresh sample procurement, sample processing, biomarker measurement, algorithmic analysis and generation of predictive measurements. B) Biophysical and molecular biomarkers are measured on live and subsequently fixed samples. C) Diagram of example biomarkers measured with single cell resolution.

SAMPLE PROCESSING & *IN VITRO* MICRO-ENVIRONMENT



Live cells are harvested from fresh samples. A) Biopsy/surgical samples are collected and processed into single cell cultures. B) Extracellular matrix (ECM) formulations are used to produce a permissive environment for cell survival. C) Microfluidic device, used in conjunction with ECM to promote cell survival, automates and standardizes biomarker measurement. D) Growth curve of cells derived from patient sample shows cells are available for analysis on day 2.

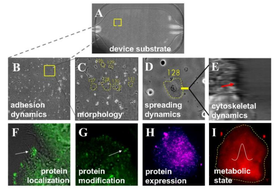


The goal of this blinded study was to demonstrate proof of principle and complete analytical validation of a diagnostic platform in prostate cancer.

To accomplish these goals, Cellanyx:

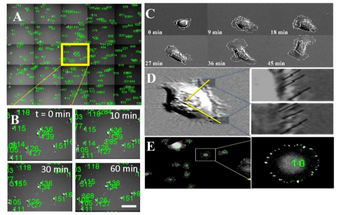
1. **Collaborated with six clinical sites across the United States.**
2. **Obtained patient informed consent and institutional review board approval.**
3. **Procured samples from excised radical prostatectomy (n=237), field study (n=67), specimens.**
4. **Received fresh/live samples shipped overnight at 4°C**
5. **Cultured the live tissue-derived cells on a microfluidic device.**
6. **Analyzed the cells for biomarkers within 72 h of sample collection.**
7. **Computed predictive metrics characterizing each patient's sample.**
8. **Unblind the data and calculated sensitivity and specificity of diagnostic's predictive power.**

AUTOMATED BIOMARKER IMAGING



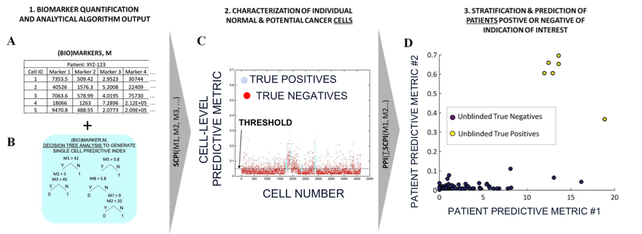
Phenotypic, biophysical, and molecular biomarkers are measured in a standardized microfluidic environment. A) Cell growth chamber coated with ECM. Biomarkers measured include B) cell adhesion rate to device substrate, C) cellular morphology, D) rate of cell spreading on substrate, E) rapid dynamics of the membrane surface, F,G,H) expression, localization, and phosphorylation state of subcellular protein complexes and individual proteins, I) and metabolic activity. 20x DIC and 40x fluorescence images were measured via a standard automated fluorescent microscope

AUTOMATED SINGLE CELL IDENTIFICATION, TRACKING, & BIOMARKER QUANTIFICATION



Machine vision algorithms are used to identify and track individual cell's biomarkers. A) Cells are identified and tagged with unique IDs B) Cell location is tracked over time. C) Cells' spreading dynamics are tracked automatically. D) Membrane fluctuations are measured to capture cytoskeletal dynamics. E) Subcellular protein complexes and protein activation states are automatically identified and measured on fixed, fluorescently stained cells.

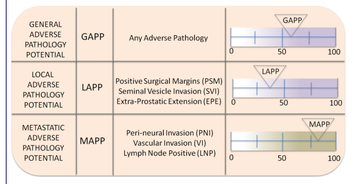
MACHINE LEARNING ALGORITHMIC ANALYSIS OF LIVE BIOPSY CELLS TO STRATIFY PATIENTS



Cellanyx's Machine Learning algorithm has the ability to process multiple biomarkers and accurately predict various pathological outcomes. (A) A set of biomarkers measured for each cell in a patient are input to (B) Cellanyx's machine learning algorithm that generates multiple decision trees stratifying cells of a negative patient from cells of a positive patient for a given pathological outcome. The decision trees are weighted to optimize algorithm accuracy. (C) A representative plot demonstrating stratification among negative and positive cells utilizing combinations of biomarkers as described by the decision trees. Patient level results are obtained by summarizing cell level results into (D) A (representative) plot demonstrating stratification of patients for a given predicted pathology finding.

CLINICAL RESULTS

CELLANYX'S CLINICAL METRICS



Clinical Highlights

1. Sensitivity and specificity numbers describe the capability of proprietary* prostate cancer diagnostic test to predict adverse pathologic findings.

2. The General Adverse Pathology Potential (GAPP) describes the possibility of any pathologies from either lesion or 'field samples.' Similarly, Local Adverse Pathology Potential (LAPP) describes the extension of tumor in the prostate capsule and seminal vesicles, and the Metastatic Adverse Pathology Potential (MAPP) describes invasion into peripheral systems such as blood, lymph and/or bone. The GAPP, LAPP, & MAPP calculation is made with a proprietary* algorithm.

$$\text{sensitivity} = \frac{\text{true positives}}{\text{(true positives + false negatives)}}$$

$$\text{specificity} = \frac{\text{true negatives}}{\text{(true negatives + false positives)}}$$

Note that not every sample tested had the same pathology data reported and 'N' values represent the number of patients with a given adverse pathology reported yes or no.

PREDICTIVE PERFORMANCE FROM PROSTATE TISSUE SAMPLES

Metric	Sensitivity	Specificity	AUC	Sample N=
Lymphnode Positive	0.91667	0.97175	0.94492	189
Seminal Vesicle Invasion	0.89655	0.78894	0.86796	228
Positive Surgical Margin	0.81159	0.83234	0.86518	236
Extra Prostatic Extension	0.83333	0.8626	0.88961	221
Lymph-vascular Invasion	1	0.79703	0.9024	223
Perineural Invasion	0.87117	0.89744	0.9414	202
BCR	1	1	1	61
GAPP	0.8359	0.88095	0.87729	237
LAPP	0.79661	0.78992	0.84845	237
MAPP	0.76923	0.89706	0.88649	237

PREDICTIVE PERFORMANCE FROM PROSTATE TISSUE 'Field Effect' SAMPLES

Metric	Sensitivity	Specificity	AUC	Sample N=
Lymph node positive	1	0.93878	0.94558	52
Seminal Vesicle Invasion	1	1	1	63
Positive Surgical Margin	0.88889	0.87234	0.88534	65
Extraprostatic Extension	0.96296	0.87179	0.90883	66
Lymph-vascular Invasion	1	1	1	65
Perineural Invasion	0.92308	1	0.97788	62
GAPP	0.91228	1	0.96316	67
LAPP	0.87879	0.88235	0.90553	67
MAPP	0.88679	1	0.94474	67

Conclusion

- **Proprietary* phenotypic (cellular, molecular, and biophysical) biomarker panel in living cells obtained from fresh tumor tissue is strongly predictive of adverse pathology after radical prostatectomy (RP) specimens.**
- **Proprietary* predictive metrics derived from either direct tumor OR from surrounding 'field effect' area, LAPP and MAPP, differentiate prostate cancer patients with low and intermediate grade disease based on adverse pathology and tumor behavior.**
- **Proprietary* biomarkers were predictive of adverse pathologic findings in RP specimens. LAPP was predictive of tumor burden and MAPP of metastatic potential. GAPP was predictive of adverse pathology from either lesion or 'field samples'.**
- **This novel quantitative and actionable phenotypic biomarker panel has potential utility in risk stratification and predicts adverse pathology from biopsies that do not contain tumor.**
- **This extended proof of concept clinical study in prostate cancer strongly supports future risk stratification validation studies in prostate cancer as well as other tumors (genito-urinary and other).**

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* Cellanyx Diagnostics, LLC CONTACT: INFO@CELLANYX.COM