A Rapid Methodology for Biomarker Evaluation & Validation: Application to Prostate Cancer Screening

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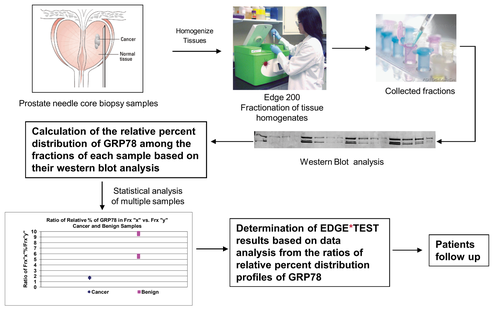
Introduction

Biomarkers have expanded across a broad range of applications from research tools during early discovery to surrogate endpoints in the clinic. Due to the development of advanced technologies, the number of publications on experimental biomarkers has expanded each year. However, only a small number of these markers will achieve clinical success. Biomarker evaluation and validation constitute critical steps in biomarker development. We have developed a rapid method, EDGE*TEST, for biomarker evaluation and validation using our Edge™ technology, a stepwise density based extraction technology in combination with a unique statistical algorithm. In this study, we used EDGE*TEST to evaluate the apoptosis marker GRP78 as an indicator and predictor of prostate cancer (PCa), and compared its effectiveness to other traditional clinical diagnostics for PCa.

Method

- Twenty-four (24) patients with elevated PSA, abnormal DRE and/or elevated PCA-3 *plus* genetic test were chosen by their urologist for this study.
- Twelve TRUS-guided needle core biopsies, six from the right gland and six from the left gland were collected for pathological evaluation by a single urologist. PSA and PCA-3 *plus* analyses were also done on each of the patients at time of biopsy.
- Under informed consent from the patient, an extended procedure was performed by the urologist on the patient. One additional biopsy from the right gland and another from the left gland were taken from random locations for EDGE*TEST.
- EDGE*TEST samples from each patient were collected into ice cold buffered saline, combined, rinsed and stored at -80°C.
- Samples were thawed and homogenized using a glass dounce. Nuclei were removed and the post-nuclear supernatants were fractionated into four subcellular density fractions (8.5%, 15%, 35% and 50%) using **Edge 200 Separation System**.
- All fractions from each sample were subjected to western blot analysis using the GRP78 marker.
- GRP78 distribution profiles within each sample were statistically analyzed to determine the optimal distribution ratios between fractions.
- EDGE*TEST results of patients' distribution ratios were blinded against all clinical and pathology data.
- Negative PCa biopsy patients were followed up for 18 months to compare predictive value of EDGE*TEST to traditional screening and biopsy.

Work Flow



EDGE*TEST

EDGE*TEST relies on **Edge** technology and Prospect Biosystems' proprietary platform to statistically differentiate between diseased and healthy (or control and treated) tissue and cell samples. EDGE*TEST distinguishes between diseased and healthy samples by defining a pair of density fractions whose biomarker content varies inversely in diseased versus healthy states. The ratio of relative marker content in these fractions can be directly correlated with diseased or healthy samples.

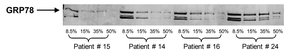
Edge technology is a non-denaturing front end sample fractionation methodology, comprising a stepwise extraction of a particle mixture using extracting media of increasing densities. It is compatible with all down stream analyses including gel electrophoresis, HPLC, mass spectrometry, and microarrays. Using Edge technology, lysates and homogenates of cell- and tissue-based samples, extracts of subcellular particles retain the biological connectivity of their components.

The EDGE*TEST method has been shown to be applicable to a broad range of diseases and dysfunctions with significant benefits.

Benefits:

- ✓ *Rapid characterization of differences between diseased and healthy tissue*
- ✓ *Identification and evaluation of predictive, diagnostic and prognostic disease biomarkers*
- ✓ *The method is INDEPENDENT of sample amount*
- ✓ *The method DOES NOT require internal or external standards*
- ✓ *Each sample is analyzed in its ENTIRETY for a defined marker*

Statistical Analysis of GRP78 Distribution



Example of Western Blots of GRP 78: all fractions were subjected to western blot analysis based on standard procedures.

Patient	Arbitrary Units of GRP78 in Each Density Fraction				Relative % Distribution of GRP78 in Each Density Fraction				Ratio of Relative % of GRP78 in Frx 8.5% vs Frx 15%
	8.5%	15%	35%	50%	8.5%	15%	35%	50%	
A	2498	466	583	261	65.60	12.24	15.31	6.85	5.36
B	1175	122	177	305	66.05	6.86	9.95	17.14	9.63
C	2006	340	138	0	80.76	13.69	5.56	0.00	5.90
D	3267	911	0	0	78.20	21.80	0.00	0.00	3.59
E	2468	1514	368	158	54.75	33.58	8.16	3.50	1.63
F	8142	3420	821	855	61.50	25.83	6.20	6.46	2.38
G	1556	159	135	0	84.11	8.59	7.30	0.00	9.79
H	2767	1096	471	109	62.28	24.67	10.60	2.45	2.52
I	3384	745	1331	936	52.91	11.65	20.81	14.63	4.54
J	912	156	0	0	85.39	14.61	0.00	0.00	5.85
K	8610	5894	4049	4834	36.82	25.20	17.31	20.67	1.46
L	3810	591	0	0	86.57	13.43	0.00	0.00	6.45
M	1460	535	514	1443	36.94	13.54	13.01	36.51	2.73
N	2142	227	189	0	83.74	8.87	7.39	0.00	9.44
O	3910	1076	312	297	69.88	19.23	5.58	5.31	3.63
P	2002	1114	1046	736	40.87	22.74	21.36	15.03	1.80
Q	4476	1129	387	0	74.70	18.84	6.46	0.00	3.96
R	913	160	0	0	85.09	14.91	0.00	0.00	5.71
S	6450	2693	686	285	63.41	26.89	6.85	2.85	2.36
T	8034	1978	810	757	69.38	17.08	7.00	6.54	4.06
U	8787	3841	1731	1611	55.02	24.05	10.84	10.09	2.29
V	4687	830	296	159	78.48	13.90	4.96	2.66	5.65
W	1483	397	64	0	76.29	20.42	3.29	0.00	3.74
X	4745	1285	568	189	69.91	18.93	8.37	2.78	3.69

* Western blot analysis was done by ImageJ software. The relative percent distribution of GRP78 in each fraction has been calculated by dividing the arbitrary units of a fraction by the total arbitrary units of all fractions within the sample.

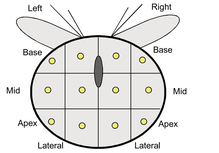
** A specific ratio (Frx 8.5%/ Frx 15%) of the relative percent of GRP78 in each sample was selected based on a statistical analysis.

References:

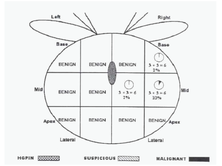
1. Lee, A. S, "GRP78 Induction in Cancer: Therapeutic and Prognostic Implications" *Cancer Res.* **2007**, 67(8): 3496-3499.
2. Fu, Y.; Wey, S.; Wang, M.; Ye R., ect., "Pten null prostate tumorigenesis and AKT activation are blocked by targeted knockout of ER chaperone GRP78/BiP in prostate epithelium" *Proceedings of the National Academy of Sciences* **2008,** 105:19444-19449.

Clinical Data and Pathological Results

Patient #	DRE*	PSA ** ng/ml	PCA-3 *Plus****	Diagnosis based on Biopsy Histology	Number of Cancerous Cores	Gleason Score	% Cancer/ core
1	Abnormal	4.80	42.40	Benign			
2	Abnormal	1.54	40.60	Benign			
3	Normal	5.40	39.80	Benign			
4	Normal	4.58	97.00	Benign			
5	Normal	4.90	60.50	Benign			
6	Abnormal	2.46	2.50	Benign			
7	Normal	2.41	25.10	Benign			
8	Normal	5.64	10.20	Benign			
9	Normal	4.46	5.10	Benign			
10	Normal	4.20	22.30	Benign			
11	Normal	4.56	9.30	Benign			
12	Normal	12.10	4.00	Benign			
13	Normal	5.70	3.10	Benign			
14	Normal	5.34	9.70	Benign			
15	Normal	5.10	6.20	Benign			
16	Normal	8.13	9.90	Benign			
17	Normal	5.80	14.30	PCa	2	6	5-10
18	Abnormal	>1500	55.60	PCa	12	7	5-90
19	Abnormal	24.70	91.70	PCa	11	7, 9	20-90
20	Abnormal	3.50	128.40	PCa	8	6, 7	6-80
21	Normal	6.20	104.10	PCa	7	6	2-85
22	Abnormal	2.30	15.00	PCa	3	6	10-60
23	Normal	5.10	9.50	PCa	3	6	2-10
24	Abnormal	6.10	51.40	PCa	3	6, 7	30-90



Twelve directed biopsies ○ in specific areas of the prostate were collected for pathological evaluation. Needle cores are taken from the right and left sides of the prostate gland near the apex, near the base, and from the central portion of the prostate gland. An additional core (not shown) was randomly taken from each side of the prostate for this study.



Prostate Biopsy Map: Pathological evaluation result from one of the PCa patients.

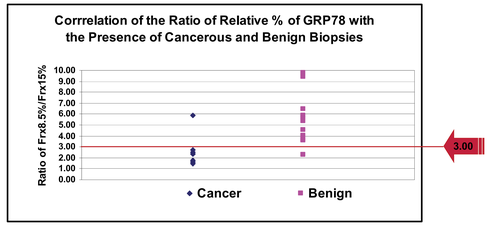
Yellow area above corresponds to those samples judged by pathological analysis to contain varying amounts of cancerous tissue.

* **DRE**: Digital Rectal Exam is a physical examination to check the prostate gland for growths in or enlargement of the gland, and to assess its size, shape, and consistency. A tumor in the prostate can often be felt as a hard lump.

** **PSA**: Prostate-specific antigen blood test. PSA is a protein produced by the prostate and released in very small amounts into the bloodstream. PSA levels under 4 ng/mL typically are considered "normal," whereas results over 10 ng/mL are considered "high". Results between 4 and 10 ng/mL are usually considered "intermediate."

*** **PCA-3 *plus***: A gene-based test that detects mRNA expression and is carried out on a urine sample. A result of a PCA-3 *plus* value of 35 or greater suggests a high likelihood of prostate cancer.

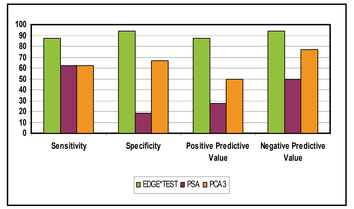
EDGE*TEST Results



Graph shows the clustered distribution of the ratios of relative percent distribution of GRP78 in fraction 8.5% vs. fraction 15% in a group of patients with cancerous and benign biopsies. The ratio of 3.00 indicated by the red arrow is an arbitrary cut off; a ratio less than 3.00 indicates cancer and a ratio above 3.00 indicates benign.

Comparison of EDGE*TEST with PSA and PCA-3 *plus* Within the Given Sample

EDGE*TEST Predictability Based on the Initial Biopsy



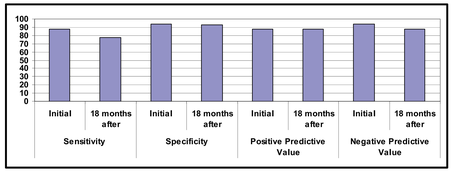
EDGE*TEST vs. Traditional Tests	EDGE* TEST	PSA	PCA3
Sensitivity	87.50	62.50	62.50
Specificity	93.75	18.75	66.66
Positive Predictive Value	87.50	27.77	50.00
Negative Predictive Value	93.75	50.00	76.92
Likelihood Ratio Positive Test	14.00	0.76	1.87
Likelihood Ratio Negative Test	0.133	2.000	0.562

Sensitivity = TP/(TP+FN);
Specificity = TN/(TN+ FP)
Positive Predictive Value = TP/(TP+ FP)
Negative Predictive Value = TN/(TN+ FN)
Likelihood Ratio Positive Test = Sensitivity/(1 - Specificity)
Likelihood Ratio Negative Test = (1 – Sensitivity)/Specificity

TP = True Positive
FP = False Positive
TN =True Negative
FN = False Negative

Diagnostic Review Guideline: EDGE*TEST <3.00, cancer; PSA: >4, cancer; PCA3: >35, cancer

Comparison of EDGE*TEST Predictability at 18 Months vs. at Initial Biopsy



- Based on initial biopsy, EGDE*TEST had a false positive of 1/16 (6.25%) with PCa not detected yet on 18 months follow up.
- Of the initial 15 patients correctly diagnosed by EGDE*TEST with benign disease and not cancer, only one patient had developed cancer on subsequent biopsy.

Conclusions

- **EDGE*TEST** predictability based on the initial biopsy is significantly superior to traditional tests.
- After 18 months follow-up, **EDGE*TEST** continues to show high predictive data of benign disease and not cancer.
- **EDGE*TEST** only needs two needle biopsies, and provides more sensitivity and more specificity than the traditional PSA test and the genetic PCa3 test.
- Changes in subcellular relative distribution of GRP78 in prostate tissue may be used as a predictor for prostate cancer.
- Changes in relative levels of apoptosis in prostate tissue may be used as a predictor for prostate cancer.
- GRP78 could be used as a potential biomarker of tissue based prostate cancer diagnostics.
- **EDGE*TEST** provides (1) a simple and reproducible technique for subcellular fractionation of tissue homogenates/cell lysates, (2) a unique statistical method for evaluation of potential biomarkers, and (3) the opportunity for generating data for further clinical validation.
- This is a preliminary data. A larger patient pool is needed for further evaluation of the method.

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