Evaluation of GRP 78 as a Potential Prostate Cancer Biomarker from Needle Core Biopsy Tissue Samples Following a Step-Wise Density Based Subcellular Fractionation

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Introduction

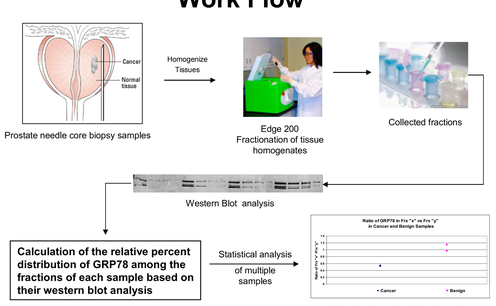
Prostate cancer (PCA) is one of the major health problems in the developed world and is the second leading cause of cancer death in men in the United States. Currently, there are two major problems inherent in the management of prostate cancer: (1) the lack of specificity of the prostate-specific antigen (PSA) test, and (2) the lack of a method to distinguish between progressive and indolent diseases. Therefore, more sensitive and more specific marker or markers than PSA are critically needed to improve and change the management of prostate cancer.

It has been reported that glucose-regulated protein GRP78 is related to several human cancers[1]. A recent study has demonstrated that GRP78 plays a crucial role in the development of prostate cancer by promoting cancer cell proliferation, mediating oncogenic signaling and protecting cancer cells against cell death resulting from the stress of tumor development [2]. GRP78 was evaluated as a potential prostate cancer biomarker to further develop a diagnostic test for prostate cancer screening. We have developed a rapid method, EDGE*TEST, to evaluate the marker in needle core biopsy tissue samples, using our Edge™ (***Enhanced density gradient extraction***) technology, a step-wise density based extraction technology. This method 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.

Method

- Twenty-four (24) patients suspected of being positive for PCA, as determined by prior clinical evaluation of their PSA and/or PCA3*plus* scores, as well as by digital rectal examination, were chosen by their urologist for this study.
- Under a standard prostate biopsy clinical procedure, a total of 12 needle core biopsies, six from the right gland and six from the left gland were collected for pathological evaluation. PSA and PCA3*plus* analyses were also done on each of the patients.
- 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 this study.
- 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.
- Results have been analyzed statistically to give relative distribution ratio profiles of GRP78 for each patient.

Work Flow



Edge Technology

Edge Technology - Enhanced **d**ensity **g**radient **e**xtraction - is a step-wise density based extraction method.

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. In lysates and homogenates of cell- and tissue-based systems, extracts of subcellular particles retain the biological connectivity of their protein components.

The technology can be accelerated and enhanced by various external forces, such as centrifugal, magnetic, electrical force, etc. When the process is driven by centrifugal force for the rapid, ordered extraction of substances, the process is called Centricollation™

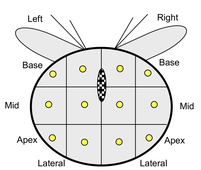
Centricollation = Edge + Centrifugal force

Benefits:

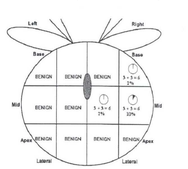
- *Significant enrichment of low-abundance proteins prior to analysis*
- *Rapid characterization of differences between diseased and healthy tissue*
- *Identification and evaluation of predictive, diagnostic and prognostic disease biomarkers*
- *Acceleration and improvement of outcome prediction using surrogate biomarkers*
- *Data arising from the process is internally referenced and DOES NOT need any standard*

Clinical Data and Pathological Results

Patient #	DRE*	PSA ** ng/ml	PCA3 *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.



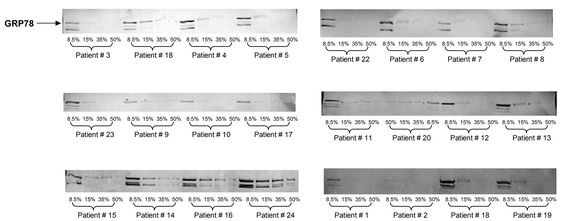
Prostate Biopsy Map: Pathological evaluation result from one of the PCA patients.

*** 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."

***** PCA3 *plus***: It is a gene-based test that detects mRNA expression and is carried out on a urine sample. A result of a PCA3 plus value of 35 or greater suggests a high likelihood of prostate cancer.

Statistical Analysis of GRP78 Distribution Data



Western Blots of GRP 78: All fractions were subjected to western blot analysis based on the standard procedure.

Patient #	Arbitrary Units of GRP78				Relative % Distribution of GRP78*				Ratio of Relative % of GRP78 in Frx 8.5% vs. Frx 15%**
	8.50%	15%	35%	50%	8.50%	15%	35%	50%	
1	2142	227	189	0	83.74	8.87	7.39	0.00	9.44
2	913	160	0	0	85.09	14.91	0.00	0.00	5.71
3	1556	159	135	0	84.11	8.59	7.30	0.00	9.79
4	4687	830	296	159	78.48	13.90	4.96	2.66	5.65
5	3267	911	0	0	78.20	21.80	0.00	0.00	3.59
6	4476	1129	387	0	74.70	18.84	6.46	0.00	3.96
7	1483	397	64	0	76.29	20.42	3.29	0.00	3.74
8	3910	1076	312	297	69.88	19.23	5.58	5.31	3.63
9	1175	122	177	305	66.05	6.86	9.95	17.14	9.63
10	912	156	0	0	85.39	14.61	0.00	0.00	5.85
11	2498	466	583	261	65.60	12.24	15.31	6.85	5.36
12	3810	591	0	0	86.57	13.43	0.00	0.00	6.45
13	4745	1285	568	189	69.91	18.93	8.37	2.78	3.69
14	8034	1978	810	757	69.38	17.08	7.00	6.54	4.06
15	3384	745	1331	936	52.91	11.65	20.81	14.63	4.54
16	8787	3841	1731	1611	55.02	24.05	10.84	10.09	2.29
17	1460	535	514	1443	36.94	13.54	13.01	36.51	2.73
18	8142	3420	821	855	61.50	25.83	6.20	6.46	2.38
19	6450	2693	686	285	63.41	26.89	6.85	2.85	2.36
20	2002	1114	1046	736	40.87	22.74	21.36	15.03	1.80
21	2468	1514	368	158	54.75	33.58	8.16	3.50	1.63
22	2006	340	138	0	80.76	13.69	5.56	0.00	5.90
23	2767	1096	471	109	62.28	24.67	10.60	2.45	2.52
24	8610	5894	4049	4834	36.82	25.20	17.31	20.67	1.46

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

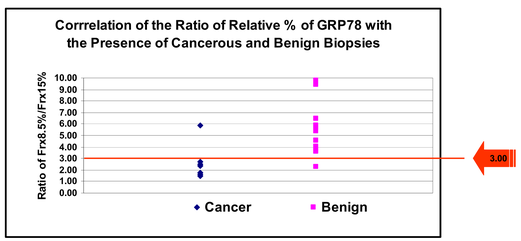
* Western blots analysis was done by ImageJ software. The relative % distribution of GRP78 in each fraction has been calculated by dividing the arbitrary units of a fraction by the total arbitrary units of all fractions within the sample.

** A specific ratio (Frx 8.5%/ Frx 15%) of the relative % of GRP78 in each sample was selected based on a statically analysis of all the ratios of possible combination of fractions.

References:

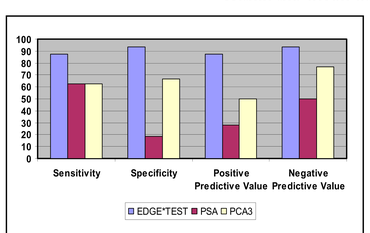
1. Lee, A. S, "GRP78 Induction in Cancer: Therapeutic and Prognostic Implications" *Cancer Res.* **2007**, 67(8): 3496-3499.
2. Fu, Y.; Wey, S.; Wang, M.; Ye R., ect., "Pten null prostate tumorigenesis and AKT activation are blocked by targeted knockout of ER chaperone GRP78/BiP in prostate epithelium"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

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.

Comparison of the Benefits of EDGE*TEST with PSA and PCA3 *plus* Within the Given Sample



Results from 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

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

For the above:
Sensitivity = TP/(TP+FN) Positive Predictive Value = TP/(TP+ FP) Likelihood Ratio Positive Test = Sensitivity/(1 - Specificity)
Specificity = TN/(TN+ FP) Negative Predictive Value = TN/(T+ FN) Likelihood Ratio Negative Test = (1 – Sensitivity)/Specificity
Where TP=True Positive, FP=False Positive, TN=True Negative and FN=False Negative

Conclusions

- GRP78 was evaluated as an indicator of prostate cancer marker, using **Edge** technology and a propriety statistical analysis method.
- Ratios of the relative percent of the marker between two specific fractions within a sample are different for patients having cancerous and benign biopsies, with tight clustering of data for the cancer samples.
- The ratio of the relative percent of the marker between two specific fractions can be used to determine if the patient will show cancerous or benign biopsies.
- The **EDGE*TEST** method—which is based on **Edge** technology, DOES NOT require internal or external standards.
- Since the method relies on the ratio between internal fractions of the sample, the method is independent of sample amount.
- The **EDGE*TEST** method only needs two needle biopsies, and provides more sensitivity and more specificity than the traditional PSA test and the genetic PCA3 test.
- This is a preliminary data. A larger patient pool is needed for further evaluation of the method.

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