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Signal pathway profiling of prostate cancer using reverse phase protein arrays

Reverse phase protein arrays represent a new proteomics microarray technology with which to study the fluctuating state of the proteome in minute quantities of cells. The activation status of cell signaling pathways controls cellular fate and deregulation of these pathways underpins carcinogenesis. Changes in pathway activation that occur between early stage prostatic epithelial lesions, prostatic stroma and the extracellular matrix can be analyzed by obtaining pure populations of cell types by laser capture microdissection (LCM) and analyzing the relative states of several key phosphorylation points within the cellular circuitry. We have applied reverse phase protein array technology to analyze the status of key points in cell signaling involved in pro-survival, mitogenic, apoptotic and growth regulation pathways in the progression from normal prostate epithelium to invasive prostate cancer. Using multiplexed reverse phase protein arrays coupled with LCM, the states of signaling changes during disease progression from prostate cancer study sets were analyzed. Focused analysis of phospho-specific endpoints revealed changes in cellular signaling events through disease progression and between patients. We have used a new protein array technology to study specific molecular pathways believed to be important in cell survival and progression from normal epithelium to invasive carcinoma directly from human tissue specimens. With the advent of molecular targeted therapeutics, the identification, characterization and monitoring of the signaling events within actual human biopsies will be critical for patient-tailored therapy.

Keywords: Prostate cancer / Protein microarrays / Signal transduction profiling PRO 0598

1 Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in United States males and the second leading cause of cancer death [1]. Whether due to earlier detection through more widespread application of prostate cancer screening or to more aggressive treatment of localized prostate cancer or to some other factor, mortality from prostate cancer in the United States has been declining. Additionally, the incidence of advanced disease has also been declining [2].

Local therapy, in the form of radical surgery or radiation treatment, while effective, is a source of great morbidity. Unfortunately, 5–70% of men will fail local therapy and

many of these men will progress to metastatic disease [3, 4]. Additionally, 25% of men will present with locally advanced disease or metastatic disease [5]. These men are left with few treatment options other than androgen depletion or blockade, to which the prostate tumors eventually become resistant. The limitations of current diagnostic and therapeutic options in prostate cancer make advances in molecular diagnostics and therapeutics particularly appealing for this common and deadly malignancy.

With the recent completion of the map of the human genome, the next step in molecular medicine becomes determining the function of the genes and gene products. Proteomics is an emerging field that examines the final protein product of gene expression. With advancements in molecular technologies, the ultimate goal of proteomics becomes not simply protein identification, but determination of protein function in the complex cellular environment [6].

Gene expression alone cannot determine the activation (phosphorylation) state of *in vivo* signal pathway checkpoints [7]. Aberrations in the regulation of these pathways may be a key to carcinogenesis. Cell culture systems and

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Abbreviations: EGFR, epidermal growth factor receptor; ERK, extracellular signal-related kinase; HPRC, hormone refractory prostate cancer; LCM, laser capture microdissection; MAPK, mitogen-activated kinases

animal models may not accurately reflect these changes [8]. A new proteomics technology, termed reverse phase protein arrays, has been developed which will allow the study of the dynamic proteome of human cancer [9]. Laser capture microdissection (LCM) allows for molecular analysis of pure cell populations [10]. When coupled with LCM for specimen procurement, reverse phase protein arrays allow examination of the relative states of several key phosphorylation checkpoints in pathways involved in pro-survival, mitogenic, apoptotic and growth regulation pathways involved in the progression from normal prostate epithelium to invasive prostate cancer.

2 Material and methods

2.1 Tissue samples

Radical prostatectomy specimens were obtained from seven men with clinically localized prostate cancer. Pathologic Gleason scores ranged from $2 + 3 = 5$ to $5 + 4 = 9$. Specimens were fully embedded and frozen. The specimens were sectioned using uncharged slides for microdissection.

2.2 Tissue processing and microdissection

Microdissection was carried out using direct pathological examination using a Pixcell 200 Laser Capture Microdissection system (Arcturus Engineering; Mountain View, CA, USA). Slides were stained with a modified hematoxylin and eosin protocol as previously described [9]. Slides were treated sequentially with 70% ethanol (10 s), deionized water (10 s), Mayer's hematoxylin (Sigma; St. Louis, MO, USA) (30 s), deionized water, blueing solution (Sigma), 70% ethanol, 95% ethanol, 100% ethanol (60 s), and xylene (60 s). All aqueous staining baths contain 10 mmol Complete™ protease inhibitor (Roche Diagnostics, Mannheim, Germany).

2.3 Cell lysis and cellular lysate arraying

Three to five thousand LCM shots (7.5–30 μm size, 1–7 cells/shot) were acquired for each tissue type. Microdissected cells were lysed for 30 min at 75–77°C using a 1:1 mixture of 2X SDS sample buffer and tissue protein extraction reagent (Pierce, Rockford, IL, USA) in volumes to keep protein concentrations at 4000 shots/20 μL (benign glands and stroma) and 5000 shots/20 μL (tumor). After cell lysis, samples were boiled for 8–10 min and stored at 4°C for arraying. Three nL of lysate were arrayed onto noncharged nitrocellulose slides with a glass backing (Schleicher and Schuell, Keene, NH, USA) with a pin and ring GMS 470 microarrayer (Affymetrix, Santa Clara,

CA, USA) using a 125 μm pin. Arrayed slides were prepared for staining by treating with Reblot (Chemicon, Temecula, CA, USA), followed by two washes with PBS washing buffer. They were then treated overnight with I-block (Applied Biosystems, Bedford, MA, USA) (casein solution) with constant rocking at 4°C. Slides were stored desiccated at -20°C . For estimation of total protein amounts, selected arrays were stained with Sypro Ruby Protein Blot Stain (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions and visualized on a Fluorchem™ imaging system (Alpha Innotech, San Leandro, CA, USA).

2.4 Protein microarray staining

Staining was carried out on an automated slide stainer (DAKO, Carpinteria, CA, USA) using a biontynyl-linked peroxidase catalyzed signal amplification system as per the manufacturer's recommendation and using components from the manufacturer's kit, unless otherwise indicated. Between each step arrayed slides were washed three times for five minutes each with TBS washing buffer. Endogenous biotin was blocked using the biotin blocking kit for five minutes, followed by application of protein block for five minutes, followed by primary antibody diluted with antibody diluent at a concentrations ranging from 1:50 to 1:2000 for 30 min and finally a secondary link antibody at a concentration of 1:100 for anti-mouse antibodies and 1:5000–1:10 000 for anti-rabbit antibodies. Signal amplification was accomplished by applying a streptavidin-biotin complex solution for 15 min, amplification reagent (biotinyl tyramide and hydrogen peroxidase) for 15 min, and streptavidin-peroxidase for 15 min. The arrays were stained using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen. Commercially available primary antibodies were used to probe specific checkpoints in cell signaling pathways: Phospho-PKC- α (S657) 1:50 (Cell Signaling Technology, Beverly, MA, USA); Phospho-ERK (T202/Y204) 1:1000 (Cell Signaling Technology); Phospho Akt (S473) 1:50 (Cell Signaling Technology) Phospho P38 (T180/Y182) 1:50 (Cell Signaling Technology); Phospho Stat1 (S727) 1:2000 (Upstate, Waltham, MA, USA); Phospho-GSK3- β (Y279/272) 1:50 (Biosource, Camarillo, CA, USA); biotinylated goat anti-rabbit IgG (H + L) 1:5000 (Vector Laboratories, Burlingame, CA, USA); and biotinylated rabbit anti-mouse Ig 1:10 (Dako Cytomation).

2.5 Image analysis

Stained slides were scanned individually on a UMAX PowerLook III scanner (UMAX, Dallas, TX, USA) at 600 dpi and saved as TIF files in Photoshop 6.0 (Adobe, San Jose,

CA, USA). The TIF images for antibody-stained slides and Sypro-stained slide images were analyzed with ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA) and Excel 2000 software (Microsoft, Redmond, WA, USA). For each antibody, the average pixel intensity value for the first point in each dilution curve was divided by the corresponding value from the Sypro-stained image of the slide to generate an intensity value normalized to the total protein present in each sample. The slopes of the dilution curve were plotted to ensure that the dynamic range had not been saturated. All antibody-staining calculations were then normalized to total protein fluorescent units.

3 Results and discussion

3.1 Protein microarray staining and analysis

Comparisons of Akt phosphorylation and activation in seven patient-matched tumor and normal epithelium along with matched stroma, all procured by LCM, is shown in Fig. 1. Each patient lysate is arrayed in a miniature dilution curve so that each analyte/antibody combination can be analyzed in the linear dynamic range of the system. This is critical for direct comparative analysis as subtle differences in protein phosphorylation can cause large shifts in biological outcomes.

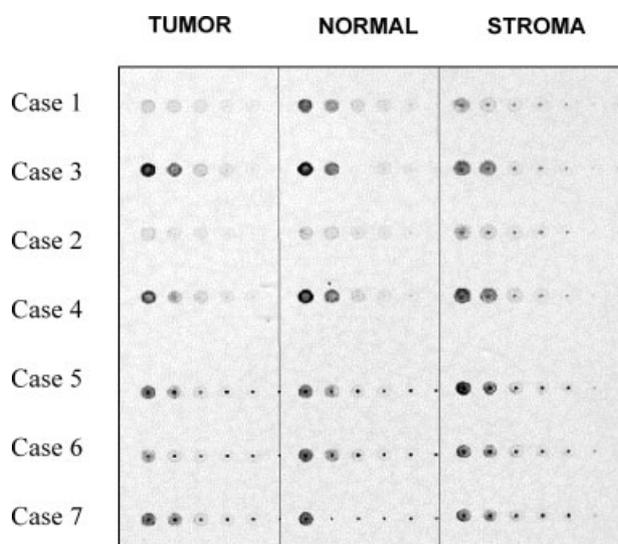


Figure 1. Reverse phase protein microarray analysis of AKT phosphorylation from seven different human prostate tissue specimens. Three-thousand to five-thousand patient-matched normal, tumor, and stromal cell procured via LCM were lysed, and arrayed on nitrocellulose slides. Each cell lysate is arrayed in a five point dilution curve of 1:2 dilutions and stained with an antibody that recognizes AKT when serine 473 is phosphorylated. The dilution proceeds from left to right with the most concentrated (neat) spot from each specimen on the far left.

We focused our analysis for this feasibility study on phosphorylation endpoints that would provide surrogate and direct evidence of the activation state of pro-survival, pro-mitogenic and cell cycle mediating pathways. Following staining, the arrays were scanned and analyzed in ImageQuant. Average pixel intensity values of the first spot in each dilution curve were normalized to the intensity values obtained for total protein staining on the array in order to compare intensity values between tumor histotypes and disease stages. Through the analysis of the slope of the dilution curve, it was determined that none of the points, including the most concentrated were at saturation. Normalization of the antibody staining was performed using the calculations for the same lysate analyzed on an identically prepared array and stained for total protein using a fluorescent stain. Histograms comparing the average normalized intensity value for each of the phospho-specific endpoint are shown in Fig. 2.

Pro-survival and pro-mitogenic pathways have been analyzed previously by our laboratory for prostate cancer using protein microarrays [9], and we were able to reproduce the earlier findings as activation of AKT increased in the transition of normal to tumor phenotype while extracellular signal-related kinase (ERK) activation was lower in tumor compared to the matched normal cells. Extension of this analysis to activation of PKC- α , Signal transducer and activator of transcription, glycogen synthase kinase 3 β (GSK3 β), and p38 revealed new findings that could point to potentially new targeted therapies. First, activation of AKT was recapitulated. Furthermore, activation of GSK3B, a known substrate of AKT, provides evidence that this pathway is activated to a higher degree in prostate cancer. The preliminary finding that activation of PKC- α is down-modulated in prostate disease is significant. Intriguingly, PKC- α activation has been implicated in controlling AKT phosphorylation in phosphatidylinositol-3-kinase (PI3K) driven events such as insulin signaling where PKC- α was found to be necessary for keeping AKT phosphorylation and activation in check as a potential negative regulator [11]. This finding provides reinforcing evidence of the importance of the AKT pro-survival pathway in prostate carcinogenesis. This could also have profound effects on the rationale behind some current therapies. A randomized phase II trial of antisense oligonucleotides ISIS 3521 examined the effects of these drugs on patients with hormone refractory prostate cancer (HRPC) [12]. ISIS 3521 is an antisense oligonucleotide directed to PKC- α . Based on our findings knocking out expression of PKC- α may be the wrong strategy for prostate cancer and would not be effective. In fact, this is exactly what was seen as 31 patients with HRPC were randomized to receive the compound and no partial or complete responders were noted in this study. This also illustrates the importance of prote-

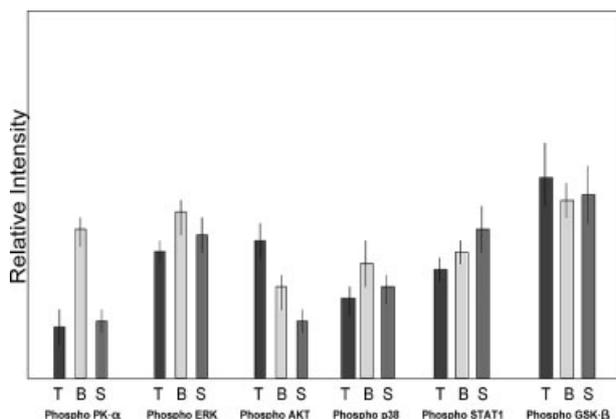


Figure 2. Signal pathway analysis of reverse phase microarrays. Analysis of phosphorylation, normalized to total protein for each spot, for six different key phosphorylation nodes of the seven specimens. Standard deviations and averages for each endpoint were calculated and shown for both normal (benign-B), tumor (T), and stroma (S).

omic technology coupled to cell signaling pathway profiling to provide new and unexpected insights into cellular processes. Furthermore, it highlights one potential advantage of the reverse phase protein array technology to examine the status of multiple signaling events simultaneously in potential patients prior to starting therapy.

4 Concluding remarks

Despite the declining mortality and incidence of advanced prostate cancer in the United States, prostate cancer remains the second most common solid malignancy in men and the second leading cause of cancer death [1]. Additionally, despite increasing efforts aimed at screening and early detection of prostate cancer up to 25% of men will have advanced disease at the time of diagnosis [5] and 5–70% of men will fail local therapy [3, 4]. It is because of these men, whose treatment options quickly become limited, and the rapid advances in the understanding of cancer at a molecular level, that the promise of novel agents aimed at the cellular mechanisms of prostate cancer are so appealing.

Management options for patients who present with advanced disease or patients who fail local therapy have traditionally been limited to waiting or hormonal deprivation. However, responses to hormonal therapy are not long-lasting and survival is limited once a patient develops HRPc. No traditional chemotherapy regimens have demonstrated a survival benefit in patients with HRPc

[13]. Increasingly, therefore, researchers and clinicians have pursued new drugs targeted to the molecular mechanisms of prostate cancer. Strategies have focused on targeting potential genes for therapy, antisense therapy, manipulation of signal transduction pathways, anti-angiogenic therapies and pro-apoptotic therapies.

Microarray technology has allowed for the identification of numerous genes that may be involved in the development and progression of prostate cancer. Dhanasekaran *et al.* used complementary DNA microarrays to examine gene expression profiles of normal, benign prostatic hyperplasia, localized cancer and metastatic HRPc in more than 50 benign and malignant human prostate cancer specimens [14]. Two genes, hepsin and PIM1 were examined at the protein level using tissue microarrays. Gene expression was correlated with clinical PSA recurrence after radical prostatectomy; PSA failure was significantly associated with absent or low hepsin immunostaining and decreased expression of PIM1 as measured by immunostaining.

Because growth factors are required for cellular proliferation and their dysregulation is thought to produce malignancy, they have become popular targets for investigation and intervention. The Epidermal Growth Factor Receptor (EGFR) has been implicated in angiogenesis, tumor growth and progression in HRPc [13]. Other related growth factor receptors include HER-2 (neu or ERB2). Multiple therapeutic strategies have been used to target the EGFR signal transduction cascade including monoclonal antibodies directed against the extracellular ligand-binding domain of the receptor, antisense oligonucleotides directed against EGFR ligands or the receptor itself, low molecular weight inhibitors of the receptor tyrosine kinase activity and low-molecular weight compounds directed against downstream components of the signal transduction pathway, such as mitogen-activated kinases (MAPK) and ERK [15].

Trastuzumab (Herceptin) is a monoclonal antibody directed against the extracellular domain of HER-2. Herceptin has been shown to have a survival benefit when combined with chemotherapy in patients with metastatic breast cancer that overexpress HER-2 [16]. A phase II trial of trastuzumab in androgen independent and dependent prostate cancer found that the drug was not effective as a single agent [17].

ZD1839 (Iressa) is an example of a low-molecular weight inhibitor of the EGFR tyrosine kinase that is orally active and has had success in preclinical studies in a wide variety of solid tumors [18]. EGFR overexpression has been demonstrated in prostate tumors and some preclinical data suggest that one mechanism for androgen inde-

pendent growth is *via* activation of the androgen receptor by non-steroid-hormonal signal transduction pathways such as the EGFR signaling pathway. Numerous pre-clinical studies have demonstrated activity of ZD1839 against prostate cancer.

Phase I studies have shown activity of ZD1839 in patients with advanced prostate cancer. Of 19 patients in phase I trials of ZD1839, 14 were evaluable for PSA response and there were 2 responders ($\geq 50\%$ PSA decrease for at least 6 weeks). Symptoms improved in 8 of 12 evaluated patients [15]. There are currently ongoing phase I/II trials of ZD1839 in combination with various chemotherapeutic agents.

Currently, low-molecular weight drugs directed specifically against downstream targets in the EGRF signal transduction cascade (MAPK, ERKs) have been identified, but have not been used clinically [19]. As the drug discovery field places greater emphasis on the development of molecular-targeted therapeutics, this study underscores the need for the precise characterization of the activity status of pathways and targets of interest in individual tumors beyond levels of gene amplification and total protein expression. Gene microarray analysis alone cannot accurately recapitulate the states of these pathways, and will be greatly complemented by proteomic analysis of the phosphorylation states of cellular circuitry. The detection of subtle changes in the activity of various signaling pathways in normal and tumor tissue in a patient is not only valuable scientifically for the study of disease progression, but will be essential for appropriate treatment selection and monitoring treatment efficacy in the future. Reverse-phase protein microarray technology provides a means to detect, in a highly multiplexed way, changes that are the direct targets for therapy. Based on these early feasibility studies, we are evaluating larger study sets of prostate cancer specimens and hope to analyze the changing state of many dozens of phosphorylation endpoints. Future goals are to employ protein microarray technology for signaling associated with aggressive vs. indolent phenotypes of prostate cancer and outcome analysis. In the future, use of protein microarray technology that can specifically analyze the protein circuitry from biopsy material before, during and after therapy may provide a

rational basis for the targeting of entire pathways using focused combinations of inhibitors selected for each patient.

Received May 23, 2003

5 References

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