Published in final edited form as:
J Proteome Res. 2009 June ; 8(6): 3044–3054. doi:10.1021/pr8009337.

Pathway Biomarker Profiling of Localized and Metastatic Human Prostate Cancer Reveal Metastatic and Prognostic Signatures

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Abstract

Reverse phase protein microarray technology was used to study key signaling pathways thought to be involved in the progression of benign epithelium to the lethal phenotype of prostate cancer. Specimens of androgen-stimulated localized prostate cancer (N=21) and androgen-deprivation therapy-recurrent local (N=4) or metastatic (N=11) prostate cancer were laser capture microdissected prior to analysis. The results showed significant increases in protein expression levels in malignant epithelial cells and patient-matched stromal tissue, which included higher levels of the apoptotic proteins Bax and Smac/Diablo and increased phosphorylation of Bcl2 (S70). The mitochondrial protein Smac/Diablo and the transcription regulatory protein STAT3 (Y705) correlated with Gleason sum and differed statistically in high Gleason grade (8-10) prostate cancers. Distinct metastasis-specific pathways were activated by caspase cleavage activation, ErbB2 phosphorylation, Bax total protein and Bcl-2 phosphorylation while phosphorylation of all three members of the MAPK family, ERK, p38 and SAP/JNK, were reduced significantly in metastatic lesions compared to primary cancers. This study, the most comprehensive pathway analysis ever performed for human prostate cancer, presents evidence of specific pathway biomarkers that may be useful for assessment of prognosis and stratification for therapy if validated in larger clinical study sets.

Keywords

prostate cancer; signaling; proteomics; protein microarray; reverse phase array; metastasis

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Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy in United States males and the second leading cause of cancer death[1]. Despite declining mortality and migration towards lower stage disease, 3.5% of men still present with locally advanced or metastatic disease [2]. Additionally, up to 35% of men presenting with clinically localized disease will fail local therapy [3, 4]. First line treatment for men who progress after failing local therapy or for those men who present with advanced or metastatic disease is most commonly androgen deprivation therapy (ADT), which is not considered curative since most cancers recur as a lethal phenotype for which most treatments are ineffective. Despite recent advances in cytotoxic chemotherapy for ADT-recurrent prostate cancer[5, 6], overall survival fro these patients remains less than 2 years. Clearly, a better understanding of the molecular pathogenesis of prostate cancer is needed along with more effective treatments for clinically aggressive and metastatic disease.

Recently, a number of publications have shown that a number of types of human solid cancers are driven by pathway derangements[7-9]. Indeed, these recent findings highlight that while mutations underpin cancer, and each patient has a seemingly distinct mutational fingerprint, at a functional level, the mutational hits coalesce into distinct pathway effects and reveal that cancer is a protein pathway disease. Advancements in molecular technologies have led to the need to determine protein function in the complex cellular environment, and how protein pathways are linked and coordinately regulate cellular function[10]. The use of protein-directed technologies is needed because gene expression analysis cannot accurately predict protein expression nor catalog the post-translationally driven activation (such as by phosphorylation) that orchestrates signaling[11-13]. Cell culture systems and animal models may not accurately reflect these changes[13]. Finally, most current therapies are directed at protein targets and these targets are often protein kinases, their substrates, or both. The activation states of these proteins and networks fluctuate constantly depending on the cellular microenvironment. Consequently, the application of molecular profiling to provide individually tailored therapy should include direct proteomic pathway analysis of patient material. Moreover, because the cellular kinome represents a rich source of new targets for molecular therapeutics, technologies that can profile and assess the activity of these molecules in human tissues are critical for realization of patient-tailored therapy[14].

A new protein micorarray technology, reverse phase protein arrays (RPMA), allows for study of protein signaling pathways[15-18] and measurement of up to hundreds of signaling proteins and their degree of phosphorylation in tissue biopsy samples.[19] Coupling laser capture microdissection (LCM) to RPMA profiling was shown recently to be a necessary for accurate analysis of malignant epithelium signaling portraits [19]. Previous publications have shown the feasibility of RPMA-driven cell signaling analysis of patient-matched stroma, malignant epithelium and normal/benign epithelium from prostate cancer patients[16]. The current study expands upon this earlier feasibility work and more thoroughly investigates stromal-epithelial cell signaling changes in the context of human prostate cancer, and investigates whether specific cell signaling changes arise in the context of aggressive disease and the metastatic microenvironment.

Materials and Methods

Tissue samples

A total of 25 prostate cancer specimens were used for this study, which resulted in 24 patient matched cases of laser capture microdissected malignant tumor, stroma and benign epithelium being procured, with one case where only malignant tumor epithelium was procured. All tissue was procured under full patient consent and IRB approval. Six frozen radical prostatectomy

(RP) specimens were received from Catholic University, Santiago, Chile and 4 transurethral resection specimens of ADT-recurrent locally advanced or metastatic prostate cancer that caused urinary retention were received from University of North Carolina, Chapel Hill, NC. Fourteen RP samples were ethanol-fixed, paraffin-embedded tissues collected at the National Cancer Institute or the National Naval Medical Center. Pathologic Gleason scores ranged from 2+3 = 5 to 5+4 = 9. Seventeen patients had Gleason sum= 4-7 and 5 patients had Gleason sum score = 8-10. Eleven metastatic cancer specimens were procured under a rapid autopsy protocol at the University of Michigan[20]. Autopsies were performed within 4 hours of death, with the average length of time between death and tissue procurement 1 hour 30 minutes. Of these 11 samples of metastases, 5 were from soft tissue, 4 from liver, 1 from bone and 1 from lung. The specimens were sectioned using uncharged slides for microdissection.

Tissue processing and microdissection

Frozen tissues were embedded in OCT prior to sectioning, and all specimens were sectioned at 8 μm onto uncharged slides for LCM. Representative sections from each case were stained with hematoxylin/eosin and reviewed by a pathologist to confirm presence of cancer. Slides were deparaffinized or ethanol-fixed and stained with hematoxylin as described previously[21, 22]. LCM of benign, stromal and malignant cells was carried out using a PixCell 2 Laser Capture Microdissection System (Arcturus Engineering; Mountain View, CA, USA). Three to five thousand LCM shots (7.5–30 μm size, 1–7 cells/shot) were acquired for each tissue type.

Cell lysis and reverse-phase array printing

Microdissected cells were lysed for 30 min at 75–77°C using a 1:1 mixture of 2× SDS sample buffer and tissue protein extraction reagent (TPER, Pierce, Rockford, IL, USA) in volumes corresponding to 3-4000 shots/20 μL (stroma and benign glands) or 5000 shots/20 μL (tumor). Cell lysates were stored at -80°C prior to printing. A total of 85 microdissected tissue lysates were separated into 2 groups and approximately 40nL of lysate was printed in duplicate onto nitrocellulose-coated glass slides (FAST Slides, Schleicher and Schuell, Keene, NH) with an Aushon 2470 solid pin microarrayer (Aushon Biosystems, Billerica, MA, USA) equipped with 325 μm pins. Samples were printed in 5 point, 1:2 serial dilution curves and 50 slides were printed for each group. Three stromal samples with sufficiently large lysate volume were included in both groups and served as bridging cases between arrays; A431 and A431 + EGF cell lysates (BD Biosciences, San Jose, CA, USA) were printed on both arrays and used to normalize data between arrays. Slides were stored dessicated at -20°C prior to staining with antibody.

Protein microarray staining

For estimation of total protein amounts, selected arrays were stained with Sypro Ruby Protein Blot Stain (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and visualized on a Kodak ImageStation (Eastman Kodak, Rochester, NY, USA). Printed slides were prepared for staining by treating with 1× Reblot (Chemicon, Temecula, CA, USA) for 15 min, followed by 2×5 min washes with PBS. Slides were treated overnight with blocking solution (1g I-block(Applied Biosystems, Bedford, MA, USA), 0.5% Tween-20 in 500mL PBS) with constant rocking at 4°C. Staining used an automated slide stainer (DAKO, Carpinteria, CA, USA) using a biontinyl-linked peroxidase catalyzed signal amplification system as *per* the manufacturer's recommendation and components from the manufacturer's kit, unless otherwise indicated as previously described [21]. Slides were stained with a set of 39 antibodies antibodies against phosphorylated or total forms of proteins involved in cell proliferation, survival, motility and apoptosis signaling. A complete list of antibodies, sources and dilutions used for these experiments is available in Supplementary Table 1. All antibodies were subjected to extensive validation for single band, appropriate MW specificity by Western

blot as well as phosphorylation specificity through the use of cell lysate controls (e.g. Hela +/- pervandate, Jurkat +/- Calyculin).

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 7.0 (Adobe, San Jose, CA, USA). The TIF images for antibody-stained slides and Sypro-stained slide images were analyzed using MicroVigene v2.8.1.0 (VigeneTech, Carlisle, MA, USA). Briefly, final data values for each sample were calculated using the factor average mode, and all values were negative control subtracted and normalized to total protein staining via SYPRO dye within the analysis. These data values were normalized to either the A431 or A431 + EGF data value to facilitate comparison of sample values between paired arrays stained with the same antibody.

Statistical Analysis

Two-way, unsupervised hierarchical clustering and one-way analyses were carried out using JMP v5.1.2. (SAS Institute, Cary, NC, USA). Statistical comparisons and correlations among various groups of samples were conducted using the R statistical software v2.7.2 (R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.)

Results and Discussion

LCM was used to procure pure populations of patient-matched tumor and benign epithelium along with stromal cells from 24 patients in order to more fully elucidate stromal-epithelial cell signaling differences in prostate (the 25th sample yielded only malignant tumor epithelium via LCM). Unsupervised hierarchical clustering analysis (Figure 1) of 38 protein signaling endpoints chosen for measurement based on their key roles in tumorigenesis revealed a high degree of patient-specific signaling. While there was definite clustering of stromal (in blue) and benign (in green) cell populations, malignant epithelial signatures appeared more diverse. However, malignant, benign and stromal signatures often clustered together, which indicates more similarity of pathway activation within a patient regardless of cell type than among the same cell type across many patients. Patient-specificity in signal profiling has been shown previously in ovarian[23] and breast[19] cancers and reinforces the concept of patient-specific therapy. Recently, analysis of stromal-epithelial signaling in colorectal cancer tissue using the same approach revealed distinct signaling changes between the stromal and malignant epithelial compartments[24].

Standard statistical mean comparison identified several pathway changes that were cell type-specific. An unsupervised clustering heatmap display of the 3 statistically significant endpoints (figure 2) showed that annexin I and II and phosphorylation of p38 (T180/Y182) are more highly expressed/activated in the normal/benign epithelial cell population than patient-matched malignant epithelial cells. Loss of expression of annexin I and II during tumorigenesis has been shown to play a direct functional role in normal cellular function of benign prostate epithelium[25-27]. The observed decrease in phopshorylated p38 in malignant epithelium compared to matched normal/benign cells warrants further exploration. Other MAPK proteins (e.g SAP/JNK, ERK) were not affected similarly so the significance of this observation in the context of tumorigenesis and the tumor microenvironement remains uncertain.

Stromal-malignant epithelial comparisons yielded a larger number of endpoints that differed statistically (Figure 3). A number of these proteins, such as Bax, Smac/Diablo, and cleaved caspase 9, are critically important for regulating apoptosis. In prostate cancer, Bcl-2 has been

found to be important in progression and resistance to treatment with ADT, chemotherapy, and radiation[28]. A high Bcl-2 to Bax ratio has been associated with resistance to radiation treatment[29]. Antisense oligonucleotide to Bcl-2 and Bcl-x_L increased the sensitivity of LNCaP cells to treatment with docetaxel and paclitaxel[28]. A number of proteins involved in EGFR signaling, including EGF and c-erbB2 receptors, downstream MEK-ERK signaling components and STAT3 signaling molecules, were more highly phosphorylated in malignant epithelium compared to stroma. EGFR family signaling is one of the most intensely studied pathways in oncology and its importance in therapeutic targeting of prostate cancer is well-known[30, 31].

Several researchers have investigated the interaction or “cross-talk” between prostate stroma and epithelium[32]. This interaction is thought to be especially important in the development of metastases, particularly bone metastases. In addition to *in vivo* animal experiments, recent research using human prostate biopsy specimens has shown that stromal pathology can be correlated with patient outcomes. Yanagisawa *et al* examined 224 human prostate biopsy specimens from patients who subsequently underwent radical prostatectomy[33]. They found that reactive stromal grading was an independent predictor of biochemical recurrence.

Thus, it appears that there are a number of significant pathway differences between different selected cell types in the human clinical study set analyzed, with malignant epithelium more similar to benign epithelium than stroma adjacent to cancer. The utility of LCM to provide this type of careful molecular profiling is revealed by such an analysis, and provides justification for a more careful field effect. Stroma nearby and far away from malignant epithelium should be analyzed so that questions concerning the potential effects of epithelium-mesenchymal transition on signaling pathways can be studied directly; such studies have been undertaken in colorectal cancer[24]. Further understanding of the signaling activation of stroma surrounding prostate cancers may increase the number of potential therapeutic targets.

No phosphoprotein endpoints were associated with pathological T-stage. Many of the localized cases were from radical prostatectomy specimens and these cases were clinically localized (cT1-T2). Many of these were demonstrated to have extraprostatic extension on final pathology; in general, in cases where the final pathologic stage was available, the T stage was between pT2b and pT3a, thus reducing the likelihood that stage-dependent variation in signaling might be found.

Pathway markers that could have direct translational/clinical value were sought by evaluating whether pathway signatures from primary malignant epithelium were similar to signaling within epithelium from distant metastasis and if pathway markers existed that correlated with Gleason score. Signaling activation portraits of LCM-procured malignant epithelium from frozen metastasis tissue samples from bone, liver, lung and soft tissue sites from 11 patients were compared to the malignant epithelium from the prostate primaries of 25 other patients. Unsupervised clustering analysis identified endpoints that distinguished metastatic epithelium from primary malignant epithelium (Figure 4). Clear separation of the metastatic epithelium was seen, regardless of site of metastasis, which was driven largely by c-erbB2 levels and phosphorylation of c-erbB2 (Y1248), Bcl-2 phosphorylation (S70), Bax, STAT 3 phosphorylation (Y705) and elevated caspase cleavage (Figure 4,5). The phosphorylation of 3 major members of the MAPK family, ERK, p38 and SAPK/JNK, was lower in malignant epithelium in metastases compared to primary sites (Figure 4,5). These metastasis-specific changes do not appear to be prognostic within this study set since no correlation with Gleason sum score was seen (Figure 5).

STAT3 phosphorylation has been associated with both tumor initiation and progression[34]. STAT3 activation has also been shown to be specifically associated with malignant

transformation of prostatic epithelial cells[35]. The transforming ability of STAT3 in cancers seems to require its constitutive activation by aberrant growth factor signaling[36]. STAT3 also correlated with high Gleason sum (8-10), although this correlation was not found in this study set.

The exact role of ErbB2 (Her2) in prostate cancer is not well understood, but is a known drug target for CaP that is under intensive investigation. Determination of Her2 status has been shown to be critically important in breast cancer treatment decisions and management, and the success in targeting the Her2 receptor in breast cancer has prompted studies of Her2 in prostate cancer. While this study is the most detailed signaling analysis of prostate cancer metastasis to date, it has been shown that total HER2 can be overexpressed in prostate cancers, which has been shown recently to correlate with metastatic potential[37, 38]. However, strategies targeting Her2 did not demonstrate the same clinical efficacy in prostate cancer as observed in breast cancer[39, 40]. Recently, her2-directed vaccine trials for prostate cancer have shown some promise[41] However, previous studies have failed to measure phosphorylated HER2 in either local or metastatic prostate cancer, and the phosphorylated level of HER2 correlated most strongly with response to herceptin therapy[42]. These results, if validated in larger study sets, suggest the potential for selecting men with metastatic prostate cancer for anti-c-erbB2 therapy based upon phosphorylated/activated her2 status.

Cell signaling analysis comparisons between specimens of primary and metastastic prostate cancer could be confounded by processing delays of the metastatic material and lack of patient-matching. Protein degradation appears an unlikely explanation for differences since protein profiles were not always decreased in metastatic compared to primary sites (figure 6). The elevation of Bax protein and caspase cleavage in malignant epithelium from metastases compared to primary tumors probably reflect real biology but could reflect apoptotic events at time of death prior to necropsy and tissue procurement. Obtaining abundant fresh prostate metastatic material is difficult because of the limited role for surgical resection in metastatic prostate cancer. Although the primary tumor is accessable for study, metastases are the lethal phenotype of this disease. If study of larger study sets reveals potential metastasis-specific signaling events, direct biopsy of metastatic sites may be justified to guide accurate therapy.

An important aspect of the study was also the careful attention to sample handling and processing for the tissue samples at each institution since all of these specimens were part of ongoing molecular profiling efforts. Each tissue, except for those collected under warm autopsy, were immediately snap frozen on dry ice within 15 minutes after procurement, or subjected to ETOH fixation. Previous publications from our laboratory whereby patient-matched frozen and ETOH-fixed paraffin embedded tissue were directly compared by proteomic analysis, have indicated that there is no discernable differences in the protein profiles between frozen and ETOH fixation [43]. While cell signaling changes within metastatic tissue could be influenced by tissue procurement differences compared to the primary tissue, our finding of increased c-erbB2 phosphorylation in metastatic lesions is supported by recent literature as previously described above. Nevertheless, our metastasis-specific pathway biomarker findings of course need to be further validated in independent study sets

Differences in signaling between the primary tumor and metastatic lesions have important therapeutic implications. As molecular markers are identified to guide therapy, reliance on analysis of pathologic material will become critical. It will be important to determine whether the signaling characteristics of metastatic lesions and the primary tumor are similar. The results suggest that there may be differences in signaling between the primary site and metastatic lesions. Previous studies in breast cancer have shown similarities in gene expression profiles between the primary tumor and metastatic sites [44]. However, other researchers have shown important differences between primary tumors and their metastases [45]. To our knowledge,

analysis of cell signaling changes between primary tumor and metastatic tumor has not been explored in prostate cancer. The treatments for each of the men, who were all castrate, whereby metastatic tissue was obtained was highly heterogeneous, with each patient receiving different duration of treatment, types of androgen deprivation therapy and chemotherapy regimens. Most patients underwent either primary or palliative radiation. Despite this heterogeneity and the location of the metastatic tissue itself, the signaling portraits of the metastatic tissue were broadly similar. However, the direct impact of the treatment itself on signaling portraits obtained is unknown since the tissue was procured at least a few months after the last therapy and in some cases a few years after the last therapy. Since no molecular targeted kinase-directed inhibitors were used on these patients, it is unclear which, if any, signaling endpoint(s) were changed in response to treatment. However, it is certainly likely that the signaling portraits were changed in the residual cells that grew back and/or survived the therapy(ies). Indeed, these metastatic lesions may be comprised of prostate cancer stem cells that have been shown to be inherently resistant to most therapies in cell culture and animal models [46]. Further work is required to understand the significance of hormonal, chemotherapy and radiation-mediated treatment effects on tumor cells in vivo as well as the potential role of prostate cancer stem cells in the metastatic process. Nonetheless, rational therapy of metastatic prostate cancer disease using molecular targeted pathway inhibitors will require an understanding of the ongoing pathway activation of those cells within the metastatic lesion, regardless of prior treatment regimens.

Finally, we sought to uncover the existence of potential prognostic pathway markers of prostate cancer aggressiveness., Smac/Diablo and phosphorylated STAT3 (Y705) were found elevated using univariate analysis in malignant epithelium from patients with histologically more aggressive cancer (Gleason sum = 8-10) compared to patients with histologically less aggressive cancer (Gleason sum = 5-7). The potential role of STAT3 activation in prostate cancer tumorigenesis was described earlier. Smac/Diablo was elevated in high Gleason score cancers that is unexpected given its known apoptosis-inducing role in cellular function. This finding will require validation, but a potential hypothesis is that compensatory signaling pathways are activated by negative feedback loop(s) as cellular control mechanisms attempt to slow down aberrant cell growth [47]. Elevation of Smac/Diablo was not noted in metastatic prostate cells compared to primary tumors, nor was it elevated in the malignant epithelium from the 4 patients with clinically advanced tumors. While increased cleaved caspase 3 and 9 levels were elevated in malignant epithelium from metastatic versus primary sites, since patients with clnically advanced tumors and patients with metastatic disease have tumors are androgen resistant, it is intriguing that the pro-apoptotic protein Smac/Diablo is lower in those cells from tumors that are androgen independent and have a more advnced phenotype. This result will also require further validation in larger cohorts of samples.

Concluding remarks

LCM can be used to procure enriched cell populations [19] that provide the opportunity to begin to elucidate cell type-specific signaling changes within the complex prostate tissue matrix and the very different microenvironments encountered at sites of metastases. With the proliferation of molecularly targeted therapeutics, the identification, characterization and monitoring of signaling events within actual human primary and metastatic tissues will be essential for patient-tailored therapy. This study represents the most comprehensive direct pathway and drug target activation analysis ever performed using prostate cancer clinical specimens with both androgen dependent and androgen independent signaling as well as prosate tumor cells within the metastatic microenvironment. Stromal and epithelial-specific signaling changes were observed and provide the basis for further mechanistic studies. The results indicate that pathway changes in LCM-procured metastatic cells transcend site of metastasis and may provide for new therapeutic strategies for metastatic disease or prevention of the development

of metastatic disease. These signaling pathway alterations were not predictable based on analysis of the malignant epithelium in the primary and indicate that effective treatment of metastatic prostate cancer using targeted kinase-directed therapies requires interrogation of the metastatic cancer itself.

Given the current limited role for tissue biopsy and surgical resection for treatment of metastatic prostate cancer, opportunities to study metastatic human prostate cancer tissues are few. As a result, little is known about metastasis-specific molecular alterations. These and other data warrant establishment of a biorepository of metastatic prostate cancer specimens. Future studies should investigate if signaling changes within the metastatic lesion are causal to the metastatic process and targetable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors appreciate the generous support of Dr. Vikas Chandhoke and the Department of Life Sciences at George Mason University. This work was partly supported by NCI-PO1-CA77739.

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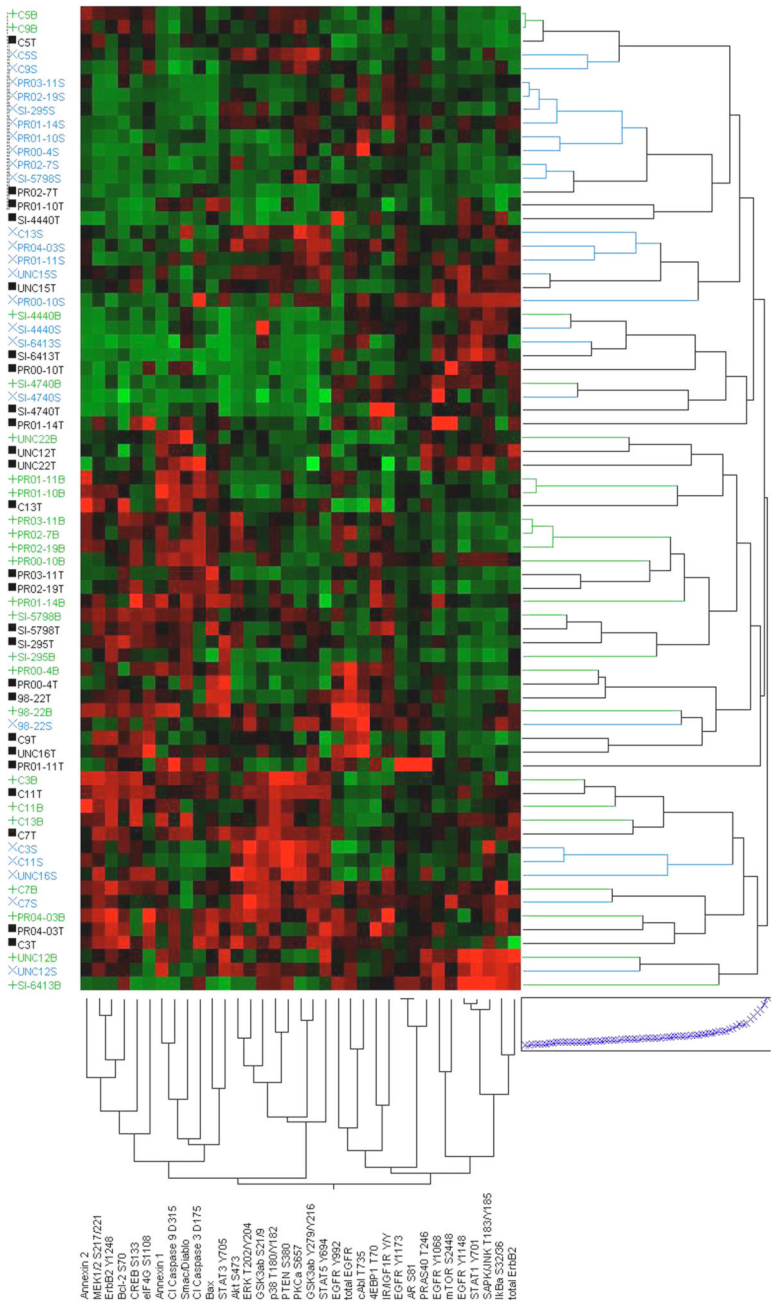

Figure 1.

Two-way, unsupervised hierarchical cluster map of microdissected, patient-matched malignant or benign epithelial and stromal cell populations. Samples are designated by case number followed by C=cancer (black), B=benign (green) or S=stroma (blue).

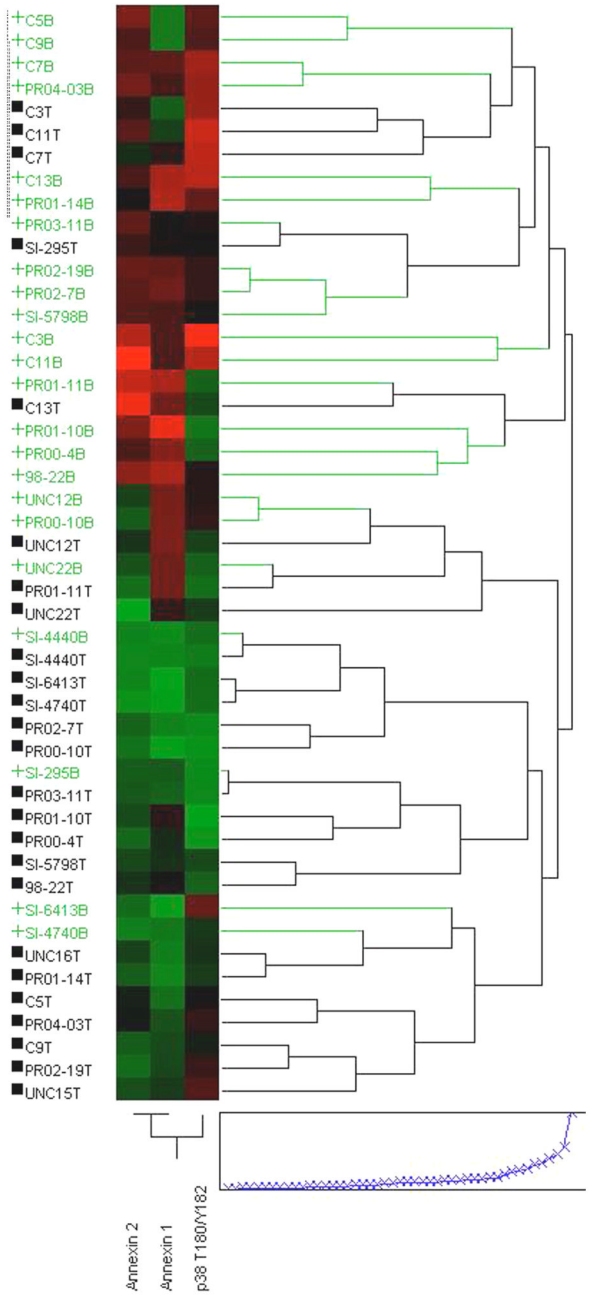

Figure 2.

Two-way, unsupervised hierarchical cluster map of statistically different endpoints between malignant and benign tissue groups. Samples are designated by case number followed by C=cancer (black) and B=benign (green).

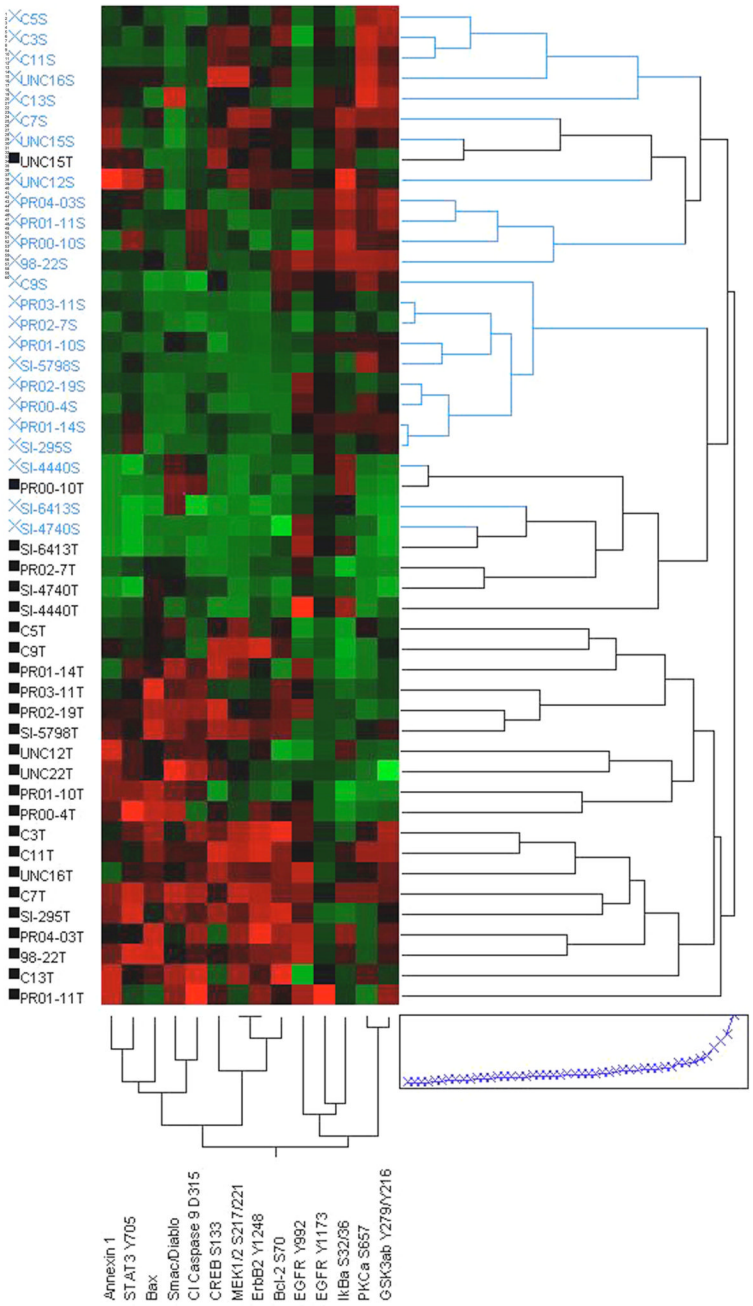

Figure 3.

Two-way unsupervised hierarchical cluster map of statistically different endpoints between malignant and stroma tissue groups. Samples are designated by case number followed by C=cancer (black) and S=stroma (blue).

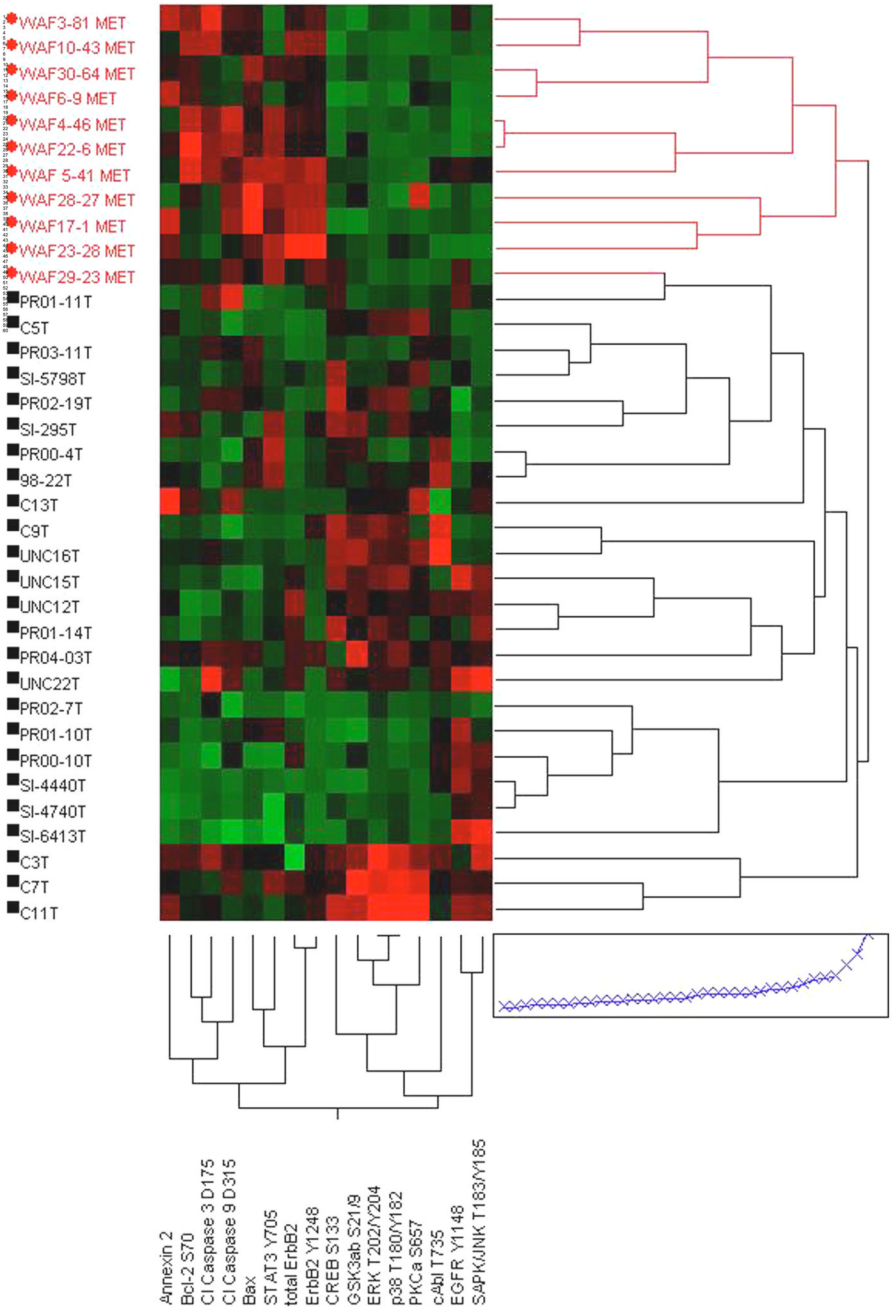

Figure 4.

Two-way unsupervised hierarchical cluster map of statistically different endpoints between malignant and metastatic tissues. Samples are designated by case number followed by C=cancer (black) and MET=metastasis (red).

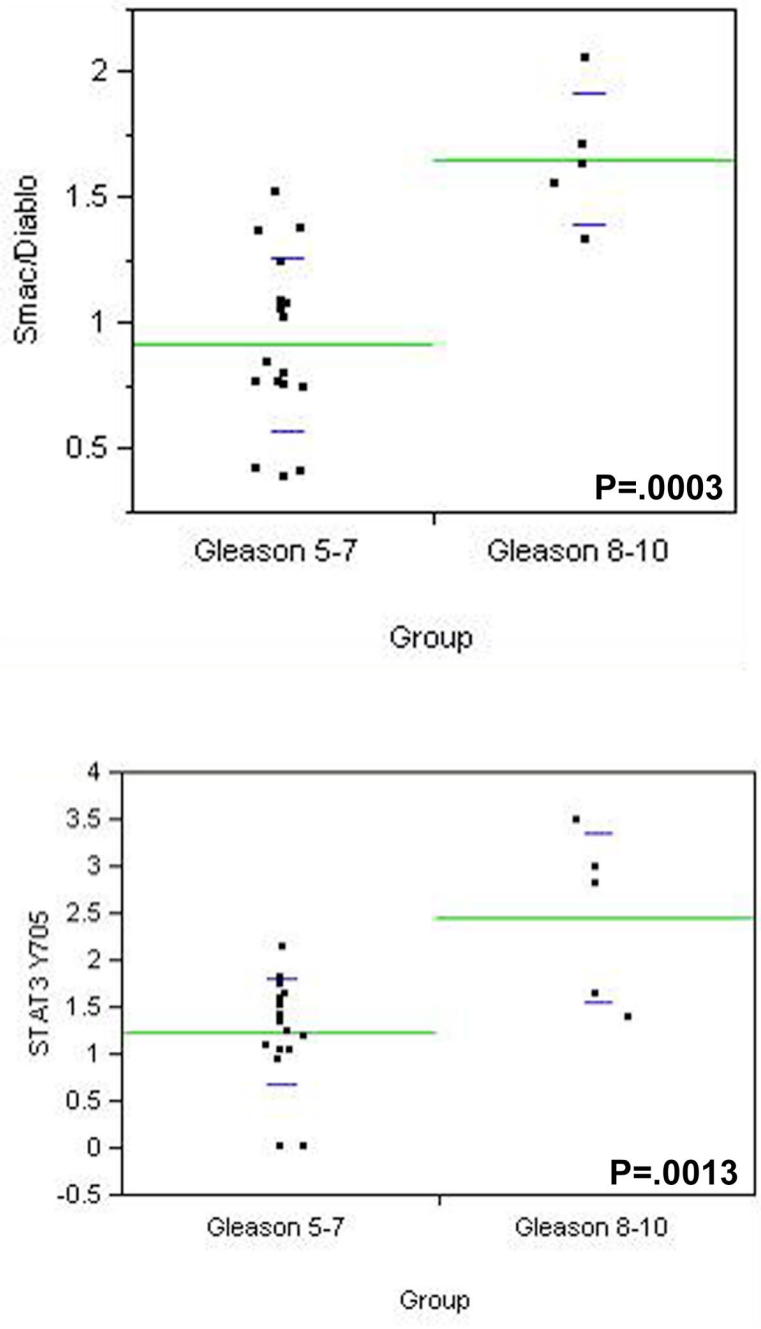

Figure 5.
One-way analyses of statistically different endpoints between Gleason sum 5-7 and Gleason sum 8-10 tumors. Means for each group are indicated by a long green line, and standard deviations are indicated by short blue lines.

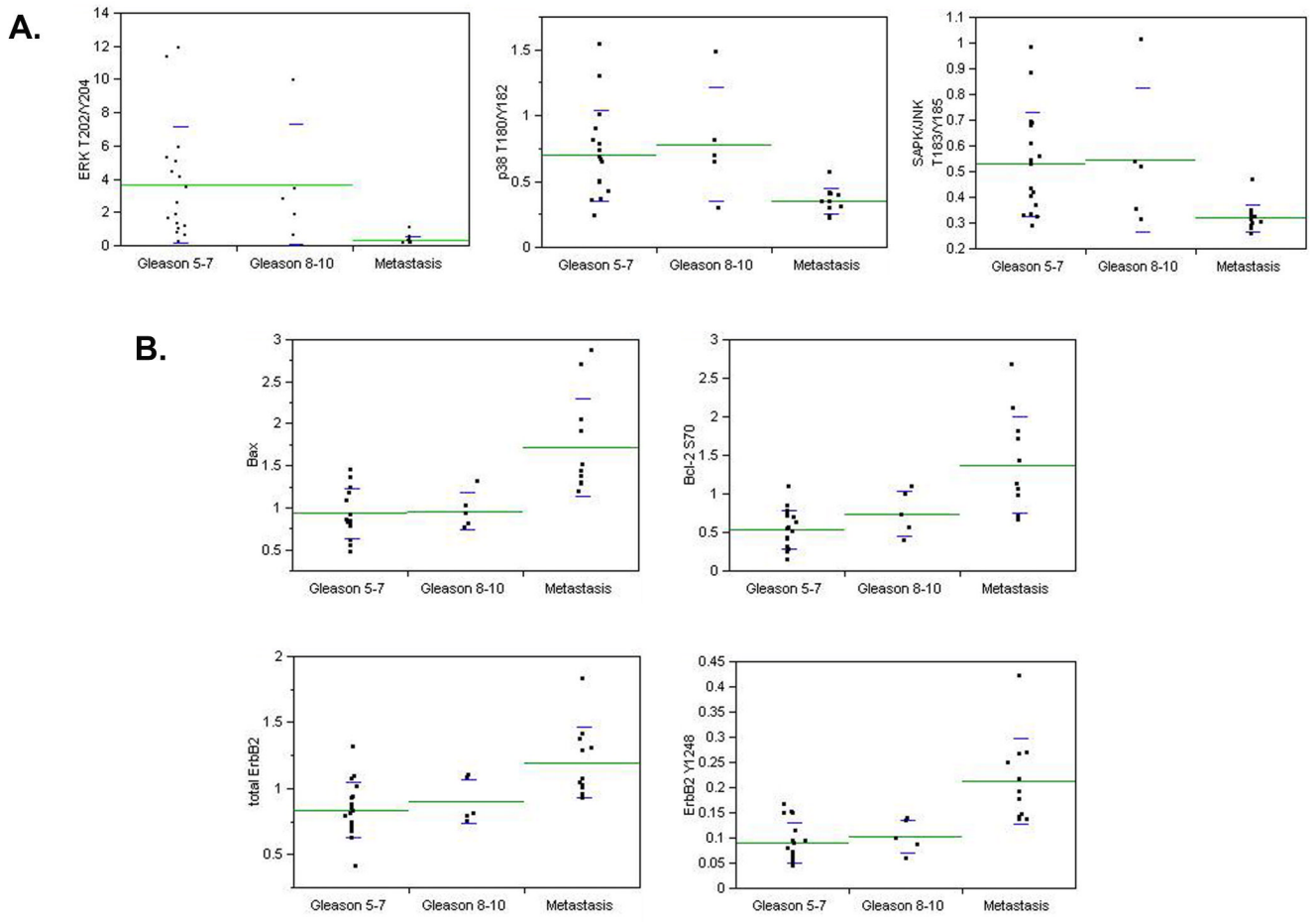

Figure 6.

One way analyses of selected statistically different endpoints between primary and metastatic sites. Cancers are separated into Gleason sum 5-7 and 8-10. **A.** Comparison of MAP kinase family activation levels between primary and metastatic site groups. **B.** Comparison of Bax, Bcl-2 S70, total ErbB2 and ErbB2 Y1248 levels between primary and metastatic site groups. Means for each group are indicated by a long green line, and standard deviations are indicated by short blue lines.