Featured Article

Clinical and Biomarker Correlates of Androgen-Independent, Locally Aggressive Prostate Cancer with Limited Metastatic Potential

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ABSTRACT

Purpose: We have identified a subset of patients exhibiting extended survival with metastases from androgen-independent prostate cancer of which the principal site of progression was the tumor primary. The purpose of this study was to evaluate the expression of selected biomarkers to characterize this subset of prostate cancer patients.

Experimental Design: A 105 core tissue microarray was constructed from primary tumor samples from 16 patients, with matched lymph node metastases in 5 cases. Immunohistochemistry was used to evaluate selected biomarkers associated with prostate cancer progression. Standard statistical methodologies were used to compute the distribution of time to progression and overall survival associations between pairs of biomarkers. Hierarchical clustering was done between groups of biomarkers, and we devised new methods to assess homogeneity of biomarker expression.

Results: The median interval from diagnosis to salvage surgery was 65 months. The profile of biomarker expression was notable for virtual absence of neuroendocrine features, high CD10, low matrix metalloproteinase (MMP)-9, high E-cadherin expression, and high membranous β-catenin. The mean proliferative index was 12.1 ± 10.1%, and the mean apoptotic index was 3.48 ± 2.22%, and there was a significant correlation between these indices. Expression of the epidermal growth factor receptor was associated with phospho-AKT and proliferative index but inversely associated with phospho-STAT3.

Conclusions: The cohort of prostate cancer patients, characterized by locally aggressive disease rather than lethal metastatic progression, was associated with a distinctive biomarker signature. The biomarker profile was, in general, more consistent with low-grade prostate cancer exhibiting local growth rather than metastatic progression. Ongoing studies will establish whether this unique subset of patients can be identified prospectively.

INTRODUCTION

In prostate cancer, therapy of the primary is typically reserved for patients with clinically localized disease. This approach for prostate cancer is based on the rationale that the outcome of patients with prostate cancer is principally determined by the behavior of metastases. This view has led oncologists to focus on the additional development of “systemic therapy.” Unfortunately, androgen deprivation, which constitutes the basis of systemic therapy, will control established metastasis on average 18 to 24 months (1, 2). Once metastatic prostate cancer ceases to respond to hormonal manipulation, median survival is 12 to 24 months (3).

An alternative strategy is to target individual anatomic sites for additional treatment to overcome potential site-specific resistance. It is noteworthy that targeting bone metastases is reported to be useful as part of an integrated, systemic therapy strategy (4–6). In addition, combining therapy of the primary with systemic therapy results in an improved survival of patients with regional disease (7, 8).

The mechanisms implicated in prostate cancer progression are being elucidated (9, 10). The ratio of a tissue matrix metalloproteinase (MMP)-9 to an adhesion molecule (E-cadherin) implicated in prostate cancer progression has been proposed as a marker of metastases (11). In addition, the presence of neuroendocrine or ductal components has also been regarded as predictive of progression or outcome (12–14). This biomarker information applied to specific patients has the potential to extend our clinical understanding of the relevant events associated with the pathogenesis of prostate cancer progression.

The majority of patients with androgen-independent prostate cancer will progress in bone. We have identified a subset of patients with metastases from androgen-independent prostate cancer of which the principal site of symptomatic progression was within the primary site. On clinical criteria, these were judged to substantially differ from the expected behavior of the cancer. Salvage surgery was done to relieve urinary symptoms. This cohort of patients with androgen-independent prostate cancer suggested, on the basis of the clinical features, that the
mechanism of invasion, metastases, and androgen-independence were not necessarily linked.

Therefore, we undertook a candidate gene approach to more fully characterize the patients. The primary purpose of this hypothesis-generating search was to more objectively characterize this subset of patients. It is assumed that this approach would lead to objective measures of progression that would prove of clinically utility.

MATERIALS AND METHODS

Patient Population. This investigation was undertaken after approval by the M. D. Anderson Institutional Review Board. We retrieved the records of 16 patients who underwent salvage surgery for symptomatic, locally aggressive androgen-independent prostate cancer at The University of Texas M. D. Anderson Cancer Center in Houston between 1994 and 2001. Clinical history, operative findings, laboratory results, and treatment effects were obtained from the charts of patients. Time to progression and overall survival were measured from time of salvage surgery until systemic progression, confirmed by radiologic imaging, or death/last follow-up, respectively. Clinical response was defined by standard clinical criteria and was based on radiologic imaging studies and assessment of clinical symptoms.

Tissue Microarray Construction. The tissue microarray was constructed with a Beecher Instruments tissue microarray apparatus (Silver Spring, MD). A total of 5, 0.6-mm diameter, replicate cores were arrayed from each of the 16 surgical specimens. Additionally, 5 cores were arrayed from matched nodal metastases in each of the 5 patients. The completed array consisted of 105 cores of tissue. An H&E stained section from each donor block was used to identify representative areas of tumor from each donor block.

Immunohistochemical Techniques. With the DAKO AutoStainer (DAKO, Carpinteria, CA) and standard 3,3'-diaminobenzidine (DAB) immunohistochemistry, the following primary antibodies were used: Bax 1:80, β-catenin 1:8,000, and epidermal growth factor receptor (EGFR) 1:750 (Zymed, South San Francisco, CA); Bcl-2 1:25, Ki67 1:100, and p53 1:1,000 (DAKO); caspase 3 1:3,000 and MMP-9 1:1,000 (R&D Systems, Minneapolis, MN); E-cadherin 1:75 and pSTAT3 1:25 (Santa Cruz Biotechnology, Santa Cruz, CA); chromogranin 1:2,000 and serotonin 1:5 (Neomarkers, Freemont, CA); CD10 1:150 (Novocastra/Vector Laboratories, Burlingame, CA); p21 1:100 (Oncogene, San Diego, CA); and pAKT 1:100 (Cell Signaling Technology, Beverly, MA). Slides were deparaffinized in xylene and then rehydrated in decreasing concentrations of EtOH. The slides were then placed in Target Retrieval Solution (DAKO), steamed for 45 minutes, and then cooled at room temperature for 20 minutes. Endogenous peroxidases were blocked with 10% H2O2/methanol for 5 minutes and then rinsed with H2O. Primary antibodies were added at the appropriate concentration and incubated at room temperature for one hour (pAkt overnight at 4°C) and then rinsed in H2O. Species-specific Envision + System (DAKO) was added and incubated for 30 minutes at room temperature and then rinsed in H2O. DAB (DAKO) was added for five minutes and then rinsed in H2O. Slides were counter-stained in hematoxylin 1:10 (Poly Scientific, Bayshore, NY) for one minute and then rinsed in H2O, dehydrated with increasing concentrations of EtOH and then xylene, and then mounted with Cytoseal (Richard Allen Scientific, Kalamazoo, MI). Corresponding positive control tissue was used for each of the biomarkers to confirm antibody specificity and sensitivity.

Tissue Microarray Biomarker Analysis. To facilitate biomarker analysis and data management, images of tissue microarray cores for each biomarker were acquired with the Bacus Labs Incorporated imaging system (BLISS; Bacus Laboratories, Inc., Lombard, IL). High-resolution images were addressed and stored in the WebSlide format. Details and advantages of this system have been described previously (15). Tissue microarray core images were hard linked to a tissue array database (16, 17), with a Bacus Labs Active X API. Clicking on a core in the virtual array simultaneously activates the corresponding image and a biomarker scoring pop-up window. The percentage of tumor cells exhibiting detectable staining was scored as 0 (no staining), 1 (up to 25%), 2 (25 to 75%), or 3 (>75%). The intensity of staining was scored as 0 (no staining), low, or high. Additionally, subcellular distribution of biomarker expression was scored (membranous, cytosolic, nuclear, or a combination thereof).

Proliferative and Apoptotic Indices. The extent of proliferation in each tissue microarray core was assessed by scoring nuclear Ki67 positivity. At least 100 tumor cells were scored for each core and the proliferative index expressed of the percentage of Ki67-positive cells. Similarly, processed (active) caspase 3 was used to extent of cell death and expressed as a percentage of positive cells in each core. Active caspase 3 was used in preference to the terminal deoxynucleotidyl transferase-mediated nick end labeling technique to estimate apoptosis, because it provided a biochemical confirmation of engagement of an activated cell death-signaling cascade.

Statistical Analysis. The endpoints to be analyzed include measurements representing the involvement or intensity of a biomarker, which can be recorded as an ordered or continuous variable, time to progression, and overall survival. Spearman’s rank correlations were calculated to quantify the strength of any meaningful associations between pairs of biomarkers. The assessment of difference in the expression of biomarkers between locations was carried out via the Jonckheere-Terpstra test for ordered alternatives. The Kaplan-Meier estimate was computed for the distribution of time to progression and overall survival. The log-rank test was applied to assess the effect of single risk factors such as stage and Gleason grade (18). Because the sample size of independent subjects was small, no multivariate regression analyses were done. In addition, the hierarchical clustering of the biomarkers was carried out to group biomarkers together according to similarity. In particular, there are initially as many clusters as biomarkers. The most similar biomarkers are first grouped, and these initial groups are merged according to their similarities. Eventually, as the similarity decreases, all of the subgroups are merged into a single cluster. All statistical analyses were done with SAS release 8.1 (SAS Institute Inc., Cary, NC) and S-PLUS 2000 (Mathsoft Inc., Seattle, WA).

To assess homogeneity in biomarker expression over the 5 cores for each patient, we used two methods: (1) The amount of
agreement was estimated via the proportion of the mode of the
equation: entropy = −∑ p_i log p_i, where
(1) the information entropy was estimated for each patient
estimates of agreement averaging around 80%.
and chromogranin were expressed at low levels or were
1.61 (total heterogeneity where all of the 5 cores are
developed androgen-independent state (Table 1). In 7 of 14 patients, chemotherapy
3. Results
Clinicopathologic features of the 16 patients with locally aggressive prostate cancer are
their potential to predict prostate cancer progression. These biomarkers included bax, bcl-2,
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in prostate cancer progression. These biomarkers included bax, bcl-2, β-catenin, CD10, chromogranin, E-cadherin, EGFR, MMP-9, p21, p53, pAkt, pStat-3, and serotonin. A composite of
extent of surgery was individualized for each patient and varied from retropubic prostatectomy to pelvic
to assay the expression of specific biomarkers. To facilitate this assessment, a tissue microarray was constructed
in Table 2. Homogeneity of multiple cores within each subject was assessed by estimating the proportion of agreement or the information entropy (Fig. 3). The two biomarkers, serotonin and MMP-9, had perfect agreement within all of the cases, whereas all of the remaining biomarkers exhibit high homogeneity with estimates of agreement averaging around 80%.
Markers of neuroendocrine differentiation, serotonin, and chromogranin were expressed at low levels or were undetectable in all of the cases analyzed. In contrast, CD10 (neutral endopeptidase), an enzyme catalyzing the hydrolytic degradation of neuroendocrine peptides, was characteristically expressed at high levels. Expression of MMP-9, implicated in prostate cancer invasion, was essentially undetectable in all of the cases, whereas E-cadherin, a mediator of intercellular adhesion, was characteristically expressed at uniformly high levels. Additionally, β-catenin was typically present at high levels and exhibited a distinct localization to the cytoplasmic membrane.
Expression of the p53 tumor suppressor protein varied considerably between patient samples (Table 2). Of the 16 cases, 5 exhibited no detectable levels of p53, 8 cases exhibited heterogeneous nuclear positivity, and in 3 cases, >75% of the tumor cells exhibited nuclear p53 protein. The cyclin-dependent kinase inhibitor, p21, either was typically not expressed at detectable levels

\[ \text{Table 1 Patient clinical and pathologic features} \]

<table>
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<th>n</th>
<th>%</th>
<th>Median (range)</th>
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<tr>
<td>PSA (diagnosis)</td>
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| Gleason score (diagnosis) | 75% of the tumor cells

Abbreviations: PSA, prostate specific antigen (ng/ml); LHRH, luteinizing hormone-releasing hormone.

Clinical Features of Locally Aggressive Prostate Cancer Cohort. All of the 16 patients had documented disease progression despite hormonal ablation. At initial diagnosis, 10 of 16 (62%) patients had metastatic disease (Table 1). Tumor metastases were confirmed by tissue diagnosis and consisted of pelvic lymph node involvement (9 of 10 patients) and one case involving metastasis to bone. The majority of primary tumors exhibited aggressive histology (Gleason score, 8 to 10 in 70%).

Over half of the patients received presurgical radiation therapy as primary local therapy, and the majority of patients received chemotherapy after developing androgen-independent prostate cancer (Table 1). All of the patients received hormonal ablation (7 orchectomy and 9 medical castration). Patients with localized disease at presentation were treated initially with primary definitive therapy (radiation therapy or hormonal ablation). The median time from initiation of hormonal ablation to androgen independence was 38 months with a range varying from 1 to 98 months. Androgen independence was defined as new sites of metastases or consecutive rises in serum prostate-specific antigen concentration. All of the patients exhibited objective evidence of progression. The median time from androgen independence to salvage surgery was 16 months with a range from 5 to 39 months.

The majority of patients (14 of 16) received cytotoxic chemotherapy on symptomatic progression to an androgen-independent state (Table 1). In 7 of 14 patients, chemotherapy resulted in symptomatic relief and/or minor radiographic response. No patient had partial remission by standard radiographic criteria. All patients had salvage surgery without major complications. The median interval from initial diagnosis to salvage surgery was 65 months and ranged from 11 to 102 months. The extent of surgery was individualized for each patient and varied from retropubic prostatectomy to pelvic exenteration. All of the patients, except 1, have been followed until present or death. During the follow-up period, 9 of 16 (56%) patients died. Of the 9 patients, 1 died of regional disease only. The remainder of the 9 patients died with complications of bone, lymph nodal, and/or liver metastases. Median progression-free survival (from salvage surgery) was 11 months, and median overall survival was 42 months. Kaplan-Meier curves indicated that there was no significant association between tumor stage or Gleason grade with either time to progression or survival (Fig. 1).

Immunohistochemical Assessment of Biomarkers with Tissue Microarray. We used immunohistochemical techniques to assess the expression of specific biomarkers. To facilitate this assessment, a tissue microarray was constructed with tissue obtained from salvage surgical specimens from all of the 16 patients. Biomarkers were selected on the basis of previous studies supporting their potential to predict prostate cancer progression. These biomarkers included bax, bcl-2, β-catenin, CD10, chromogranin, E-cadherin, EGFR, MMP-9, p21, p53, pAkt, pStat-3, and serotonin. A composite of several biomarkers is shown in Fig. 2. Additionally, rates of tumor proliferation were estimated with Ki67 and cell death with an antibody specific for processed (active) caspase 3. A summary of mean and median tumor involvement for each of the biomarkers for the individual patient samples is provided in Table 2. Homogeneity of multiple cores within each subject was assessed by estimating the proportion of agreement or the information entropy (Fig. 3). The two biomarkers, serotonin and MMP-9, had perfect agreement within all of the cases, whereas all of the remaining biomarkers exhibit high homogeneity with estimates of agreement averaging around 80%.

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Expression of the p53 tumor suppressor protein varied considerably between patient samples (Table 2). Of the 16 cases, 5 exhibited no detectable levels of p53, 8 cases exhibited heterogeneous nuclear positivity, and in 3 cases, >75% of the tumor cells exhibited nuclear p53 protein. The cyclin-dependent kinase inhibitor, p21, either was typically not expressed at detectable levels.
(7 cases) or nuclear expression was noted in <25% of the cells (4 cases). With Spearman rank correlations, there was no significant association between p53 and p21 expression ($\tau = 0.03$). Both the proapoptotic bax protein and antiapoptotic bcl-2 protein exhibited diffuse cytosolic expression in the majority of the 16 patients and exhibited the highest correlation of any pair of biomarkers assessed in this study ($\tau = 0.62$). However, bcl-2 was expressed in <25% of prostate cancer cells in three cases, and focal bax expression was observed in one of these cases. EGFR was either undetectable or expressed at low levels in 6 of 16 cases (37.5%), and an equal number of cases expressed diffusely high levels of EGFR in the prostate cancer cells.

Fig. 1  Kaplan-Meier estimation for the distribution of time to progression and overall survival corresponding to different stage and Gleason score groups. (OS, overall survival; TTP, time to progression)

Fig. 2  Immunohistochemical analysis of tissue microarray. Examples of nuclear Ki67 reactivity used to calculate proliferative indices (A), diffuse staining of E-cadherin (B), CD10 (C), and EGFR (D). Expression of MMP-9 (E) and chromogranin (F) were, in general, modest or undetectable in tumor cells.
Activation-state dependent antibodies were used to assess two signaling intermediates that have been recently implicated in the pathogenesis of prostate cancer progression, phospho-AKT (protein kinase B), and phospho-STAT3. The majority of cases (12 of 16) showed no detectable expression of phospho-STAT3, in 3 cases <25% of the cancer cells exhibited nuclear phospho-STAT3 protein, and in a single case the majority of tumor cells exhibited nuclear phospho-STAT3 positivity. The expression of phospho-STAT3 was correlated with phospho-AKT expression (r = 0.27) and inversely correlated with both β-catenin (r = 0.23) and EGFR (r = 0.4). In contrast, expression of cytoplasmic phospho-AKT was demonstrable in 15 of 16 cases. There was a moderate correlative association between phospho-AKT expression with EGFR (r = 0.21), β-catenin (r = 0.26), E-cadherin (r = 0.29), bax (r = 0.47), bcl-2 (r = 0.26), p53 (r = 0.22), and chromogranin (r = 0.28). A summary of the Spearman’s rank correlation analysis for comparisons between all of the biomarkers is provided in Table 3.

Prostate cancer proliferative indices varied considerably among the 16 patient samples between 0 and 33% with a mean of 12.1 ± 10.1%. Apoptotic indices varied from 0 to 9% with a mean of 2.48 ± 2.22%. The linear regression model was fit to assess the association between cell death and proliferation. The Pearson’s correlation coefficient of r was calculated from this model. This analysis yielded an r value of 0.58, indicating a significant correlation between the proliferative index and apoptotic index in individual cases (r = 0.019).

Hierarchical clustering analysis of biomarker expression plot also showed the homogeneity of biomarker expression within subjects (Fig. 4). Additionally, hierarchical clustering enabled a visual assessment of the pattern of biomarker expression.

Matched primary tumor and nodal metastases were represented for 5 of the 16 patients in the tissue microarray. There were no significant differences between matched primary tumor and nodal metastases in the expression of biomarkers assessed in this study.

**DISCUSSION**

This cohort of patients exhibited clinical behavior characterized by several distinctive features. Although the patients initially had metastases, substantial progression at the time of chemotherapy occurred only locoregionally and not at metastatic sites during a median follow-up of 65 months. Given these features, it was considered that a strategy incorporating aggressive therapeutic modalities targeted toward the primary tumor could potentially benefit these patients.

Markers of neuroendocrine differentiation were distinctly
absent from this androgen-independent prostate cancer cohort. This finding is in marked contrast to the well-defined acquisition of neuroendocrine features that characterizes relapsed androgen-independent prostate cancer (12–14, 19). There is consistent evidence that neuropeptides impart a proliferative and/or survival signal to prostate cancer cells after androgen deprivation (20). The availability of neuropeptides to mediate these paracrine effects is associated with a down-regulation of membrane neutral endopeptidase (CD10) on prostate cancer cells. Thus, the abundance of neuropeptides may be because of the androgen ablation-induced loss of their common catalytic enzyme, CD10 (21). Our findings are consistent with this concept in that the paucity of expression of neuroendocrine markers was associated with a high level of CD10 protein in almost all of the specimens.

Expression of bax or bcl-2 per se does not appear to be associated with the distinct clinical features of the cohort of patients examined in this study. Similarly, the frequency of apparent p53 mutations in our series is therefore similar to that reported for other cohorts of androgen-independent prostate cancer (22).

Resistance to apoptotic cell death induction directly con-

Table 3  Spearman’s rank correlations between biomarkers

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<th>β-catenin</th>
<th>Bax</th>
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tributes to multistep carcinogenesis (23, 24). The mean cell death index in our cohort, as assessed by active caspase-3 expression, varied from 1 to 9% with a mean of 3.48 ± 2.22% (median, 3.00 ± 2.24%). These rates are higher than those previously reported for patients undergoing radical prostatectomy (25). In this group, the median apoptotic index varied from 0.9% in those that exhibited disease recurrence to 0.41% in the nonrecurrent group. Although the cohort of prostate cancers examined in this study were characterized by a relatively high rate of apoptosis, this was accompanied by a corresponding elevation in proliferation. Tumors comprising our cohort exhibited a proliferative index that ranged from 1 to 33% with a mean of 12.1 ± 10.13% (median, 8.0 ± 10.8%). Similar to the apoptotic index, these results are 2-fold higher than those reported in a series of previously untreated patients undergoing radical prostatectomy (26). Linear regression analysis revealed a substantial and direct correlation between apoptosis and proliferation in the individual cases comprising this cohort. This observation is not completely unanticipated in that there is considerable evidence suggesting that cell death sensitivity and cell proliferation are linked at the molecular level (27, 28).

MMP-9 is known to mediate extracellular matrix degradation associated with tissue invasiveness in some tumor types, including prostate cancer (29). Although there was evidence of tumor dissemination in the majority of patients in this cohort, there was no case exhibiting detectable levels of MMP-9 protein. E-cadherin is a mediator of intercellular adhesion (30). Down-regulation of E-cadherin is associated with prostate cancer progression (31–33). The current cohort exhibited uniformly high levels of E-cadherin expression. Finally, we assessed the expression of β-catenin, a multifunctional protein that directly binds to the cytoplasmic tail of E-cadherin and forms a multi-protein complex with cytoskeletal structures to promote adhesion (34, 35). The subcellular distribution of β-catenin, membranous or nuclear, can provide insight into its function in adhesion or wnt signaling, respectively. In the current study, β-catenin, similar to E-cadherin, was diffusely expressed in the majority of cases and exhibited distinct membrane localization. Therefore, this cohort of clinically distinct androgen-independent prostate cancers, characterized by locoregional progression and paucity of disseminated bone marrow metastases, show no evidence of E-cadherin/β-catenin codown-regulation that has recently been suggested to contribute to metastatic progression (36). Instead, these findings are more typical of organ-confined prostate cancer (36).

The EGFR (or ErbB-1) has been shown to be an important transducer of transforming growth factor-β and epidermal growth factor mitogenic signals in prostate cancer cells (37–39). Linear regression analysis showed a significant association between the extent of EGFR expression and proliferation as assessed by Ki67 immunoreactivity (P = 0.002). There are multiple signaling intermediates downstream of EGFR including phosphatidylinositol 3'-kinase/Akt (protein kinase BPKB), and STAT3 may be important in the context of prostate cancer progression (40–42). The presence of EGFR was significantly associated with expression of phospho-Akt but inversely associated with phospho-STAT3, as assessed with activation-state dependent antibodies. The majority of tumors comprising our cohort exhibited no detectable levels of phospho-STAT3. This
finding is in marked contrast with the high frequency (82%) of constitutive activation of STAT3 reported previously (42).

There is an emerging consensus about a differential treatment strategy for patients with limited metastatic cancer (26, 43, 44). In addition to systemic therapeutic modalities, this strategy involves an attempt at resection of the primary as well as the few metastatic sites. Such a strategy has resulted in prolonged survival in colorectal cancer (45–47) and breast cancer (48) compared with traditional therapeutic approaches. Establishing a causal relationship between failure to adequately control the primary tumor and metastatic progression has proved difficult. In part, this may be attributable to the lack of informative markers able to specifically predict failure at the primary site of disease versus failure because of systemic progression.

In summary, our results suggest that a clinically distinctive group of prostate cancer patients, characterized by locally aggressive disease rather than lethal metastatic progression, is associated with a distinctive biomarker signature. This signature is characterized by lack of neuroendocrine markers (chromogranin and serotonin) and corresponding high levels of CD10. An additional feature of this cohort is the maintenance of a low invasion/adhesion index as evidenced by absence of MMP-9 and sustained high levels of E-cadherin and membranous β-catenin. This molecular profile is typically observed in prostate cancers of lower grade without metastases. Therefore, the observed biomarker profile is consistent with the clinical observation that these cancers preferentially invaded rather than progressed at bone metastatic sites as would normally be expected. These observations raise the question whether this unique subset of patients can be identified and surgical intervention justified by objective criteria. This potential is being additionally explored in a follow-up clinical trial.

REFERENCES

Clinical Cancer Research

Clinical and Biomarker Correlates of Androgen-Independent, Locally Aggressive Prostate Cancer with Limited Metastatic Potential


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