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Metastasis Initiating Cells in Primary Prostate Cancer Tissues From Transurethral Resection of the Prostate (TURP) Predicts Castration-Resistant Progression and Survival of Prostate Cancer Patients

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Abstract

BACKGROUND—We previously reported that the activation of RANK and c-Met signaling components in both experimental mouse models and human prostate cancer (PC) specimens predicts bone metastatic potential and PC patient survival. This study addresses whether a population of metastasis-initiating cells (MICs) known to express a stronger RANKL, phosphorylated c-Met (p-c-Met), and neuropilin-1 (NRP1) signaling network than bystander or dormant cells (BDCs) can be detected in PC tissues from patients subjected to transurethral resection of the prostate (TURP) for urinary obstruction prior to the diagnosis of PC with or without prior hormonal manipulation, and whether the relative abundance of MICs over BDCs could predict castration-resistant progression and PC patient survival.

METHODS—We employed a multiplexed quantum-dot labeling (mQDL) protocol to detect and quantify MICs and BDCs at the single cell level in TURP tissues obtained from 44 PC patients with documented overall survival and castration resistance status.

RESULTS—PC tissues with a higher number of MICs and an activated RANK signaling network, including increased expression of RANKL, p-c-Met, and NRP1 compared to BDCs, were found to correlate with the development of castration resistance and overall survival.

CONCLUSIONS—The assessment of PC cells with MIC and BDC phenotypes in primary PC tissues from hormone-naïve patients can predict the progression to castration resistance and the overall survival of PC patients.

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Keywords

prostate cancer patient survival; metastasis-initiating cells; dormant cells; RANK signaling; multiplexed quantum dot labeling

INTRODUCTION

One in six men in the United States will be diagnosed with prostate cancer (PC), and one in 36 will die from the disease [1]. Distinguishing indolent from aggressive PC remains an unmet medical need. As active surveillance rather than early treatment becomes accepted for managing PC patients, the need for reliable predictors of progression and survival becomes increasingly critical, especially if the use of serial transrectal biopsies is to be minimized. Our laboratory recently reported that metastasis-initiating cells (MICs), defined as a subpopulation of PC cells that can form tumors locally, can also recruit and reprogram neighboring bystander dormant or non-metastatic cancer cells (BDCs) to gain tumorigenic and metastatic competency in live mice via an activated RANK (receptor activator of NF- κ B) mediated signal network [2]. Here we tested the hypothesis that the proportion of MICs and BDCs in primary human PC tissues has prognostic value for differentiating indolent from aggressive PC. In previous studies, we found that metastatic PC displayed osteomimicry, adopting a bone-like phenotype and overexpressing many bone-like proteins [3,4] including RANK and its ligand RANKL during disease progression [2,5–11]. We demonstrated that MICs with stem cell, neuroendocrine cell, mesenchymal cell, and osteomimetic properties can recruit and reprogram BDCs to express bone-like proteins and gain metastatic potential to bone and soft tissues in animal models [2]. To gain further insight into the significance of the activated RANKL-mediated cell signaling network for cancer metastasis in the clinical setting, we sought to validate RANK pathway-associated biomarkers in this study and explore the potential use of MICs in a well-documented cohort of clinical specimens from primary PC as predictors for the progression and survival of PC patients. We employed multiplexed quantum-dot labeling (mQDL)-based quantitative histopathology at the single cell level as reported previously by our group [8,9] to analyze the MIC and BDC phenotypes simultaneously in primary PC tissue specimens from patients with known overall survival, to test their predictive utility for lethal PC progression.

MATERIALS AND METHODS

Prostate Cancer Tissue Specimens

This study included patients under the care of Dr. L. W. Jones and his associates at Huntington Medical Research Institutes, Huntington Memorial Hospital, Pasadena, California. Dr. Jones and his colleagues have followed these patients for up to 15 years. The archival patient tissue specimens from Huntington Memorial Hospital were obtained in accordance with the ethical policies and procedures of the Huntington Medical Research Institutional Review Board. The single source of patient tissues reduced variability in sample handling and processing, patient data entry, pathological diagnosis by pathologists from different institutions, and patients' ethnic backgrounds.

Clinical records were obtained from the Huntington Memorial Hospital Tumor Registry. The tissues were accrued between 1981 and 1991, generally prior to the clinical availability of PSA blood assay. A total of 44 formalin-fixed, paraffin-embedded (FFPE) tissues from patients who underwent transurethral resection of the prostate (TURP) primarily for relief of bladder outlet obstruction, and were subsequently diagnosed with PC, were used for this study under IRB# Pro 00021228. Twelve patients underwent radiation or hormonal therapy, and no patient in this series underwent subsequent radical prostatectomy. Table I summarizes the age, race, pathological Gleason score, status of distant metastasis and castration resistance, overall survival, and treatments prior to TURP for all 44 patients.

Antibodies and Reagents

Goat polyclonal antibody to neuropilin-1, a co-receptor for VEGF, (NRP1, at 1:50), C-19, sc-7239, rabbit polyclonal antibodies to RANK (1:100), H-300, sc-9072 and RANKL (1:100), FL-317, sc-9073 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibody to p-c-Met (pYpYpY1230/1234/1235 at 1:100), 44888G, was obtained from Invitrogen (Carlsbad, CA). All antibodies were diluted in 1% horse serum (Vector Laboratories, Inc., Burlingame, CA) and 20% Biotin Block Reagent (Invitrogen). Secondary antibodies were prepared in a cocktail of biotinylated antibodies to mouse, rabbit, and goat IgG (Vector Laboratories). Phosphate-buffered saline (PBS) and streptavidin-conjugated QDs at 565-, 585-, 605-, and 625 nm wavelengths as 1 μ M stock solutions were from Invitrogen. Rinse buffer or PBST (0.4% Triton X-100 in PBS, pH 7.4) was routinely used as a 3 $\times$ 5 min rinse. Streptavidin-conjugated QDs (565-, 585-, 605-, and 625 nm) were prepared as 10 nM in PBS with 6% IgG free, protease-free BSA (Jackson ImmunoResearch, West Grove, PA). The blocking reagents used were Biotin Block-1% horse serum (Vector Laboratories), 20% Biotin Block Reagent (Invitrogen) in PBS, Avidin Block-2.5% horse serum and 20% Streptavidin Block Reagent (Invitrogen) in PBS.

Multiplexed Quantum-Dot Labeling (mQDL)

To perform mQDL, a single tissue section from each study case was subjected to sequential labeling for multiple biomarkers. Each biomarker labeling started with a streptavidin block, followed by primary antibody reaction and biotinylated secondary antibody incubation, and reaction with specific streptavidin-conjugated QD. De-paraffinized and antigen-retrieved FFPE specimens were incubated in Streptavidin Block Reagent for 1 hr, followed by reaction with primary antibody in Biotin Block at 4°C overnight or at 37°C for 2 hr. After rinsing, specimens were incubated with a secondary antibody cocktail at 37°C for 1 hr and then incubated with the respective streptavidin-QD at 37°C for 1.5 hr. At the end of the incubation, the specimens were subjected to a routine 3 $\times$ 5 min rinse and mounted in 4',6-diamidino-2-phenylindole (DAPI) mounting media (Vector Laboratories) for optical imaging.

The reaction sequence of the paired antibody and QD was as follows: 1) anti- RANK antibody at 37°C, 2 hr; biotinylated secondary antibody at 37°C, 1 hr; streptavidin-QD605 at 37°C, 1.5 hr; 2) anti-neuropilin-1 antibody at 4°C, overnight; biotinylated secondary antibody at 37°C, 1 hr; streptavidin-QD625 at 37°C, 1.5 hr; 3) anti p-c-Met antibody at 37°C, 2 hr; biotinylated secondary antibody at 37°C, 1 hr; streptavidin-QD565 at 37°C, 1.5

hr; and 4) anti- RANKL antibody at 4°C overnight; biotinylated secondary antibody at 37°C, 1 hr; streptavidin-QD585 at 37°C, 1.5 hr.

Positive and negative control specimens were subjected to mQDL in parallel with the test tissue slides. ARCaP_M, an invasive bone-metastatic human PC cell line established in our laboratory [12,13], was used as a positive control. To mimic FFPE tissues, the cells were prepared as cell pellets, formalin-fixed, and paraffin-embedded. Negative controls were performed by replacing each primary antibody with species- and subtype-matched immunoglobulin prepared at the same dilution and applied to an immediately adjacent tissue section. We performed optical imaging acquisition and deconvolution or unmixing using a CRi multi-spectral camera with built-in Nuance software. The individual QD-labeled protein or cell-based biomarker intensity can be detected and quantified by inForm software (Perkin Elmer, Waltham, MA). Detailed information on mQDL was previously reported [8,9]. Metastasis initiating cells, or MICs, are defined as cells with above-median levels of cell-based expression of RANKL, NRP1, and p-c-Met. Bystander or dormant cells, or BDCs, are defined as having below-median levels of expression of RANKL, NRP1, and p-c-Met.

Statistical Analyses

The primary outcome was overall survival (OS). Variables measured were age, Gleason score, and the cell-based biomarker expression intensity of RANKL, RANK, p-c-Met, and NRP1 in the cytoplasm (C), nucleus (N), and cytoplasm plus nucleus (C +N). Biomarker measurements for each patient were the mean intensity measured from 13 images (range 7–20) taken from different tissue sites at TURP. The average number of cells analyzed was 7,106 with a range between 2,956 and 11,070. Total sample size was 44 cases.

Statistical methods are described in the relevant legends to figures and tables. The Kaplan–Meier method was used to estimate OS and a log-rank test was used to compare groups. Logistic regression was used to examine the association between biomarker intensities and the clinical status of metastasis or the development of castration-resistant prostate cancer (CRPC). The level of significance was set at 5%.

RESULTS

Table I summarizes the age, race, pathological Gleason score, status of distant metastasis and castration resistance, any radiation or hormonal therapy prior to TURP, and overall survival of the 44 patients. The mean age of the patients was 75 years (range 56–92 years). The ethnic background included 39 Caucasian-Americans, one African-American, and four whose race was undetermined. The metastasis status was 20 positive, 14 negative, and 10 data not available. The number of patients who died of other causes or were lost to follow up after diagnosis was 18 and mean survival was 50.7 months (range 19–114 months). The number of events was 26 with a mean overall survival of 63.8 months (range 11–206 months). Castration-resistant PC (CRPC), defined as disease progression documented radiographically in either surgically or chemically castrated patients, was 25 positive, 11 negative, and eight data not available. The number of patients who underwent radiation, hormonal treatment, or orchiectomy was 4, 9, and 2, respectively. We found that neither

radiation nor hormonal therapy impacted the statistically significant correlation between biomarker expression and overall patient survival (data not included).

We used correlograms to show pair-wise Pearson correlations between Gleason score and biomarker expression or between two biomarkers (Fig. 1). Although Gleason score did not correlate with the expression of biomarkers, expression of the pathway-associated biomarker pairs correlated significantly. Figure 2 shows higher expression detected by image (Panel A) and labeling intensity (Panel B) of RANK-pathway mediators, RANKL, RANK, phospho-c-Met (p-c-Met) and neuropilin-1 (NRP1) in tissues from a PC patient who survived 114 months (A, top panels) compared to a patient who survived 11 months (B, bottom panels) after first diagnosis. We used the Kaplan–Meier method to estimate overall survival and a log-rank test to compare biomarker expressions categorized in the two groups, using the median as a cutoff point. While the overall survival of PC patients does not correlate with patient age or clinical Gleason score, cell-based total RANKL, p-c-Met and NRP1 expression correlated significantly with overall survival of patients with PC ($P < 0.05$) (Fig. 3A). These results are in agreement with our previous report using a different cohort of PC tissues obtained from patients from another institution [9]. A significant correlation was also found when bio-marker expression was analyzed separately in cytoplasm or nucleus (data not included) when compared with combined cytoplasm plus nucleus.

We further analyzed the association of individual biomarker expression and the clinical status of PC metastasis and development of CRPC. The results of logistic regression showed that RANKL or NRP1 expression alone was significantly associated with the clinical status of CRPC (P -value = 0.0251 and 0.0255, respectively) but not with metastatic status. We next analyzed if the percentage of MICs or BDCs could predict cancer metastasis or the development of CRPC. MICs are defined as single cells with high, or above median, levels of expression of RANKL, NRP1, and p-c-Met. BDCs are defined as low, or below median, levels of expression of the above three biomarkers. These definitions are based on the evaluation of these gene expression profiles in experimental cell models of MICs and BDCs where we observed that MICs can recruit and reprogram BDCs and alter their phenotypes permanently, including conferring metastatic potential to BDCs in mice [2]. For each tissue specimen, we analyzed 3,000–11,000 cells and found a distribution of MICs in each specimen ranging from 0 to 2,000 cells representing 0–60% of total cells and a distribution of BDCs ranging from 100 to 10,000 cells representing 4–99% of total PC epithelial cells analyzed (Table II). We found that a high ratio of MICs to BDCs predicted poor overall survival of PC patients ($P = 0.0043$ for both; Fig. 3B). Furthermore, the percentage of MICs or BDCs analyzed on their nuclear (N), cytoplasmic (C), or combined (C + N) co-expression of RANKL, p-c-Met, and NRP1 correlated with the development of castration resistance (Table III).

DISCUSSION

We showed that the signaling pathway involving RANKL-RANK, mediated by downstream p-c-MET and NRP1, can predict the overall survival of PC patients [8,9]. The present study used TURP tissues without reference to the histopathologic grading of the primary cancer to predict the lethal progression of PC by analyzing the RANKL, p-c-MET, and NRP1

molecular markers found to contribute to prostate cancer bone and visceral organ metastases in animal models. In addition to evaluating the RANKL–RANK-mediated signaling pathway, we also assessed the relative abundance of MICs versus BDCs, shown to contribute to skeleton and soft tissue metastases upon intracardiac inoculation in animal models. Analyzing the number of MICs and BDCs, we found that a higher abundance or proportion of MICs to BDCs predicted not only survival but also the development of CRPC. Increased RANK–RANKL signaling in PC cells could enhance normal bone turnover [14–17], stem cell renewal, and differentiation [16,18,19] and these factors together contribute to prostate and breast cancer bone and soft tissue metastases [5,10,20–23]. The concurrence of these two sets of data strongly supports the conclusion that MICs, which over-express the RANK–RANKL-p-c-MET and NPR1 signaling pathway, predict the development of castration resistance and the survival of PC patients.

Correlogram analyses of a statistical relationship among the downstream signaling components, suggest that these signaling components are highly related in a statistically significant manner to each other as predictors distinguishing indolent from aggressive PC. There are several underlying reasons supporting the concept that the RANK–RANKL signaling pathway plays pivotal roles conferring aggressive human PC. 1) RANKL–RANK downstream signaling components are directly linked to a large number of transcription factors that regulate over 1,644 genes broadly classified to confer osteomimicry, epithelial-to-mesenchymal transition, stem cell and neuroendocrine cell phenotypes, and cancer growth and survival related genes in PC [2]. 2) PC cells overexpressing RANKL also transition to a more mesenchymal phenotype allowing access to blood circulation through increased cell motility, migration and invasion, and subsequently enter into a metastatic environment in bone and soft tissues. RANKL-producing PC cells could elicit positive interactions with osteoclasts in bone to greatly increase their bone colonizing capability through increased osteoclastogenesis [2]. 3) Since a stem cell or neuroendocrine phenotype confers PC cell resistance to host immune defenses or chemotherapy-induced cytotoxicity [24–26], PC cells with the MIC phenotype and increased stem cell or neuroendocrine cell properties have growth advantages at both primary and metastatic sites, with an increased propensity for developing chemotherapeutic resistance or escaping immune surveillance for preferential survival [27–29]. At the present time, it is speculative to state the origin of MICs and their relationship to the development of castration resistance in PC patients. Since the clinical specimens were collected from most hormonal naïve patients, derivation of MICs, therefore, could be age-related and influenced by the genetics and hormonal status of the patients. Because our data suggest that this unique subpopulation of MICs preferentially exists in PC patients with defined castration resistance, analysis of the phenotypic expression of MICs and BDCs could hold prognostic promise for differentiating aggressive from indolent PC.

Our studies showed that the mQDL technique has the unique capability of detecting multiple bio-markers at the single cell level on the same clinical specimen, with the advantages of reduced sample variability and conservation of scarce clinical materials. The inForm software allows cell-based gene expression quantification for profiling cell characteristics, and is perfectly suited for the analysis of MICs and BDCs. Our ultimate goal is to customize

antibody-QD by conjugating them directly, reducing the labor intensive protocol and streamlining future sample analyses.

In summary, our study of clinical specimens from hormone naïve patients suggests that MICs with activated RANK-RANKL signaling, mediated by downstream p-c-MET and NRP1, confer castration resistant lethal progression of human PC. We found the proportion of MICs and BDCs predicts the status of castration resistance and the survival of PC patients. Future studies are needed to determine the etiology and evolution of MICs and BDCs in the prostate gland to gain further insights of the meta-static processes and therapeutic resistance in PC patients.

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Fig. 1.

Correlogram with pairwise correlations between Gleason score and RANK-mediated biomarker expression or between biomarkers involved in RANK-mediated signal network. The main diagonal has the covariate name. At the horizontal and vertical intersection of each covariate, the Pearson correlation coefficient (center) and the associated P -value (top right corner, ** $P < 0.01$, *** $P < 0.001$) are shown. Although Gleason score did not correlate with any biomarkers, there are significant correlations between most of the biomarker pairs.

Fig. 2. mQDL assessment of differential biomarker protein expression in clinical primary prostate cancer specimens. A. Unmixed mQDL images of RANKL, RANK, p-c-Met, and NRP1 protein expression from tissues of two patients with contrasting survival data. Scale bar, 200 μ m. B. Quantification of protein expression of biomarkers using the inForm software.

Fig. 3.

Log-rank test of: A. overall survival by age, Gleason score and biomarker protein expression in cytoplasm (C) plus nucleus (N) categorized in two groups, high and low, using the median as a cutoff point. Although age and Gleason score did not significantly correlate with overall survival, RANKL, p-c-Met, and NRP1 correlated significantly with overall survival. B. Overall survival of prostate cancer patients by number of MICs and DCs.

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TABLE I

Clinical Characterization of the Prostate Cancer Patients

Case	Age (year)	Gleason Score	Event ^a (month)	Others ^b (month)	Mets ^c	Therapy prior to TURP			
						CR ^d	Rad ^f	HT ^g	ORCH ^h
1	66	9	32		Yes	Yes	Yes	No	No
2	77	7	77		Yes	NA	No	No	No
3	76	9	61		Yes	Yes	No	No	No
4	72	8	116		Yes	Yes	No	No	No
5	76	8	19		No	Yes	No	No	No
6	69	3	39		Yes	Yes	No	No	No
7	85	10		70	NA	No	No	No	No
8	79	8	33		Yes	Yes	No	No	No
9	82	8		20	NA ^e	NA	No	No	No
10	60	7	81		Yes	Yes	No	No	No
11	66	6	73		Yes	Yes	No	No	No
12	83	8	11		NA	Yes	No	Yes	No
13	61	5	94		Yes	Yes	No	No	Yes
14	73	9	62		No	Yes	No	No	No
15	56	9	45		Yes	Yes	No	No	No
16	80	8	34		No	Yes	No	No	No
17	57	7	34		Yes	Yes	No	No	No
18	77	7	48		Yes	Yes	No	No	No
19	74	8	51		No	NA	No	No	No
20	72	5	16		No	NA	No	No	No
21	85	8	104		NA	Yes	No	Yes	No
22	67	9	25		Yes	Yes	No	Yes	No
23	84	7	80		Yes	Yes	No	Yes	Yes
24	84	7	74		Yes	Yes	No	Yes	No
25	69	6		41	NA	No	No	No	No
26	86	7	49		Yes	Yes	No	No	No
27	78	5		114	No	No	No	No	No

Case	Age (year)	Gleason Score	Event ^d (month)	Others ^e (month)	Mets ^c	Therapy prior to TURP			
						CR ^d	Rad ^f	HT ^g	ORCH ^h
28	88	4		68	NA	NA	No	No	No
29	77	6		77	NA	No	No	No	No
30	58	7		82	No	No	No	No	No
31	69	6		37	No	NA	No	No	No
32	68	3		32	No	NA	No	No	No
33	77	5		41	Yes	No	No	No	No
34	90	10		43	NA	Yes	No	No	No
35	82	8		22	NA	No	No	No	No
36	74	6		53	No	No	No	No	No
37	75	5		23	No	No	No	No	No
38	70	6		31	NA	NA	No	No	No
39	92	6		19	No	No	No	No	No
40	69	9		86	No	No	Yes	No	No
41	80	7		53	No	Yes	No	Yes	No
42	81	7	206		Yes	Yes	Yes	Yes	No
43	72	9	82		Yes	Yes	No	Yes	No
44	71	8	114		Yes	Yes	Yes	Yes	No

^aPatients died from prostate cancer since diagnosed.

^bPatients died from other causes or lost to follow up since diagnosed.

^cMetastasis.

^dCastration resistance.

^eNot available.

^fRadiation therapy.

^gHormonal therapy.

^hHormonal therapy by orchiectomy.

TABLE II
Distribution and Percent of Prostate Cancer Cells Expressing RANK-Mediated Pathway Biomarkers

Case	N ^a	p-c-Met		RANKL		NRPI		MICs ^c		BDCs ^d	
		Number ^b	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
1	8,730	33	0.38	3,646	41.76	3,185	36.48	23	0.26	4,182	47.90
2	5,608	52	0.93	1,763	31.44	2,613	46.59	48	0.86	2,683	47.84
3	5,297	140	2.64	963	18.18	1,176	22.20	113	2.13	3,724	70.30
4	6,592	202	3.06	621	9.42	194	2.94	5	0.08	5,646	85.65
5	4,108	350	8.52	654	15.92	80	1.95	8	0.19	3,115	75.83
6	6,427	439	6.83	670	10.42	153	2.38	2	0.03	5,257	81.80
7	5,933	72	1.21	7	0.12	8	0.13	0	0.00	5,851	98.62
8	3,372	1,575	46.71	2,329	69.07	2,757	81.76	1,490	44.19	448	13.29
9	7,336	11	0.15	2,298	31.32	52	0.71	4	0.05	5,035	68.63
10	6,478	471	7.27	6,210	95.86	5,507	85.01	460	7.10	250	3.86
11	6,093	796	13.06	1,326	21.76	1,258	20.65	390	6.40	4,108	67.42
12	7,330	1,352	18.44	4,844	66.08	4,627	63.12	1,164	15.88	1,808	24.67
13	6,137	1,594	25.97	4,417	71.97	4,622	75.31	1,207	19.67	788	12.84
14	6,477	2,873	44.36	4,426	68.33	2,735	42.23	2,083	32.16	1,805	27.87
15	9,639	957	9.93	2,872	29.80	1,143	11.86	510	5.29	6,416	66.56
16	8,163	4,216	51.65	2,441	29.90	520	6.37	314	3.85	3,028	37.09
17	9,038	2,392	26.47	3,576	39.57	1,345	14.88	1,098	12.15	5,019	55.53
18	8,975	292	3.25	42	0.47	33	0.37	9	0.10	8,640	96.27
19	7,717	40	0.52	102	1.32	4	0.05	0	0.00	7,576	98.17
20	9,127	2,208	24.19	1,341	14.69	1,211	13.27	269	2.95	5,648	61.88
21	7,455	150	2.01	935	12.54	1,471	19.73	10	0.13	5,299	71.08
22	6,855	108	1.58	926	13.51	1,226	17.88	17	0.25	5,184	75.62
23	4,325	1,367	31.61	3,889	89.92	2,833	65.50	1,289	29.80	349	8.07
24	7,544	77	1.02	125	1.66	445	5.90	1	0.01	6,969	92.38
25	6,879	284	4.13	211	3.07	110	1.60	7	0.10	6,340	92.16
26	10,408	128	1.23	153	1.47	3	0.03	0	0.00	10,152	97.54
27	9,720	331	3.41	758	7.80	313	3.22	201	2.07	8,879	91.35

Case	N ^a	p-c-Met		RANKL		NRPI		MIC ^c		BDC ^d	
		Number ^b	Percent	Number	Percent	Number	Percent	Number	Percent	Number	Percent
28	8,808	11	0.12	234	2.66	42	0.48	0	0.00	8,541	96.97
29	6,628	660	9.96	212	3.20	1	0.02	0	0.00	5,910	89.17
30	8,639	102	1.18	185	2.14	459	5.31	14	0.16	8,006	92.67
31	4,205	4	0.10	258	6.14	43	1.02	1	0.02	3,918	93.17
32	2,956	2,191	74.12	2,588	87.55	2,380	80.51	1,817	61.47	134	4.53
33	6,042	3,123	51.69	5,287	87.50	2,886	47.77	2,236	37.01	531	8.79
34	6,760	272	4.02	201	2.97	38	0.56	4	0.06	6,283	92.94
35	8,013	927	11.57	70	0.87	71	0.89	30	0.37	7,046	87.93
36	8,860	2,032	22.93	316	3.57	116	1.31	3	0.03	6,469	73.01
37	9,403	164	1.74	5	0.05	5	0.05	0	0.00	9,229	98.15
38	7,205	150	2.08	3	0.04	66	0.92	0	0.00	6,989	97.00
39	6,905	18	0.26	497	7.20	65	0.94	2	0.03	6,364	92.17
40	3,835	2	0.05	439	11.45	194	5.06	0	0.00	3,233	84.30
41	11,074	4,727	42.69	1,401	12.65	887	8.01	183	1.65	5,338	48.20
42	9,348	27	0.29	66	0.71	8	0.09	4	0.04	9,267	99.13
43	6,815	2,286	33.54	5,694	83.55	3,377	49.55	1,822	26.74	1,020	14.97
44	7,034	97	1.38	368	5.23	110	1.56	5	0.07	6,519	92.68

^aTotal number of cells analyzed.

^bNumber of cells positive for biomarker.

^cMetastasis initiating cells, high in RANKL, p-c-Met and NRPI.

^dBystander dormant cells, low to none in RANKL, p-c-Met and NRPI.

TABLE III
Association of Percent MICs and BDCs With the Status of Castration Resistance in PC Patients

	Positive ^a			Negative ^b			P-values
	Mean	STD	Mean	Mean	STD		
MICs (N)	7	11	3	8	0.0267		
BDCs (N)	57	31	79	25	0.0323		
MICs (C)	8	12	4	13	0.0274		
BDCs (C)	61	32	87	25	0.0152		
MICs (C+N)	8	13	4	11	0.0278		
BDCs (C+N)	58	32	83	25	0.0235		

Castration resistance status: patients with (^a) or without (^b) castration resistance.
MICs and BDCs analyzed by biomarker subcellular localization: N, nucleus; C, cytoplasm (see Materials and Methods).