Genomic alterations indicate tumor origin and varied metastatic potential of disseminated cells from prostate-cancer patients

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ABSTRACT

Disseminated epithelial cells can be isolated from the bone marrow of a far greater fraction of prostate-cancer patients than the fraction of patients who progress to metastatic disease. To provide a better understanding of these cells, we have characterized their genomic alterations. We first present an array comparative genomic hybridization method capable of detecting genomic changes in the small number of disseminated cells (10-20) that can typically be obtained from bone-marrow aspirates of prostate-cancer patients. We show multiple regions of copy-number change, including alterations common in prostate cancer, such as 8p loss, 8q gain, and gain encompassing the androgen-receptor gene on Xq, in the disseminated cell pools from 11 metastatic patients. We found fewer and less striking genomic alterations in the 48 pools of disseminated cells from patients with organ-confined disease. However, we identify changes shared by these samples with their corresponding primary tumors and prostate-cancer alterations reported in the literature, evidence that these cells, like those in advanced disease, are disseminated *tumor* cells (DTCs). We also demonstrate that DTCs from patients with advanced and localized disease share several abnormalities, including losses containing cell-adhesion genes and alterations reported to associate with progressive disease. These shared alterations might confer the capability to disseminate or establish secondary disease. Overall, the spectrum of genomic deviations is evidence for metastatic capacity in advanced-disease DTCs and variation in that capacity in DTCs from localized disease. Our analysis lays the foundation for elucidation of the relationship between DTC genomic alterations and progressive prostate cancer.

INTRODUCTION

The metastatic potential of a tumor is presumably contained within cells that are able to escape and disseminate from the primary lesion. Consequently, the identification of cytokeratin-positive cells (i.e., cells of epithelial origin) in the peripheral circulation and bone marrow of patients with clinically localized adenocarcinoma of the prostate has generated much interest. These cells are hypothesized to originate from the primary tumor and represent a very early stage in the metastatic process (1). The presence of these putative disseminated tumor cells (DTCs) in the bone marrow is particularly noteworthy, since bone metastases are a well known feature of lethal metastatic prostate cancer (2). It seems likely that these cells are the source of metastatic disease in those patients who develop secondary tumors years after resection of the primary tumor. Thus, DTCs have become a key target for risk-association studies and experiments characterizing molecular features that underlie the phenotypic traits responsible for survival, dormancy, and ultimately proliferation in environments distinct from their origin.

There is a compelling need for improved ways to evaluate patients with clinically-localized prostate cancer for their risk of developing metastasis. Currently, risk is estimated by the pathological features of the primary tumor (e.g., Gleason Score and tumor stage) and PSA levels in peripheral blood (3, 4). These predictive features are used to stratify patients into low- and high-risk groups. However, up to 50% of patients classified as high risk for progression do not develop metastasis, and ~10% of patients classified as low risk for progression subsequently develop secondary disease (5). Thus, the need to more accurately distinguish those men who would benefit from aggressive adjuvant treatment regimes from those who could be safely treated by active surveillance is a powerful motivation to improve upon the ability to predict outcome.

To date, some studies have found a positive correlation with the presence of DTCs and risk of relapse following primary therapies for localized prostate cancer (6-10), while others have not (11-16). Among the latter studies, Pfitzenmaier et al. (2007) found that 75% of pre-surgery

patients without clinical evidence of metastatic spread, and only 11% of normal controls, harbored disseminated epithelial cells in their bone marrow. This frequency far exceeds the expected frequency of biochemical recurrence or metastasis. These results strongly suggest that the mere presence of DTCs is not indicative of risk of disease progression. Why do so many patients possess DTCs, if escape from the primary tumor is a rate-limiting step in the metastatic process? Despite the low incidence in normal controls, it is possible that the disseminated cells are not actually tumor cells. Characterization of the molecular features of disseminated cells might resolve the question of their tumor origin and provide a better understanding of their relationship with disease progression.

Low-resolution and low-throughput methods such as fluorescence *in situ* hybridization (FISH) (17) or conventional comparative genomic hybridization (CGH) (18) have provided some evidence that DTCs from epithelial cancers possess genomic abnormalities. However, the molecular characterization of DTCs by high-resolution techniques has been hampered by the small number of DTCs obtainable: typically fewer than 50 epithelial cells from bone-marrow aspirates are available for analysis. To date, high-resolution genomic analysis was only possible by first generating cell lines from DTCs (19).

Given the proposed role that DTCs play in systemic disease and the paucity of genomic analyses of these cells, we have used array CGH to produce high-resolution genomic profiles of cells expressing epithelial markers isolated from the bone marrow of prostate-cancer patients. We categorize our samples by the status of the patients from which they were acquired. Thus, our samples of disseminated cells obtained from patients with organ-confined (localized) disease are designated as LocDCs. Our samples of disseminated cells from patients with metastatic (advanced) disease are referred to as AdvDCs. Our study of genomic changes in LocDCs and AdvDCs lays the groundwork for a better understanding of the role these cells play in carcinogenesis.

MATERIALS AND METHODS

Sample acquisition. All samples were collected from patients undergoing treatment at the University of Washington Medical Center or the VA Puget Sound Health Care System after informed consent was obtained. A total of 59 prostate-cancer patients (11 patients with advanced disease and 48 patients with localized disease), participated in this study. Supplemental Table 1 gives the Gleason grade and TNM stage for 55 of the patients and chemical castration status for all advanced patients. Clinical data on 4 localized patients was unavailable. The mean (SD) age of these patients was 60.2 (7.4) years. Our use of human samples for this study was approved by the Institutional Review Boards of the participating institutions.

Just prior to radical prostatectomy, bone-marrow aspirates were collected from the upper iliac crest of all patients with localized disease. Bone-marrow aspirates were collected from advanced patients prior to any treatment for four patients and during the course of treatment for seven patients. Pools of 10-20 epithelial cells were isolated and collected into 10 μl H_20 based on expression of EpCAM (CD326; a pan-epithelial cell antigen) from the aspirates of each of 58 patients and from three biological replicates from the aspirate of one metastatic patient, as described previously (14). Twenty of the 59 samples (14 from localized- and 6 from advanced-disease patients) were collected using 1:20 dilutions of both an anti-EpCAM(CD326) antibody conjugated with a fluorescein isothiocyanate isomer (Dako, Carpinteria, CA) and an anti-CD45 antibody conjugated to R-phycoerythrin (Dako) to label normal hematopoietic cells. This dual detection system was used to exclude normal lymphocytes from disseminated-epithelial-cell pools and to collect samples of normal-cell samples. These samples were analyzed by array CGH and used to define thresholds of loss and gain in disseminated-cell arrays (see Supplemental Methods and Results) and in proof-of-principle tests (see Results).

Prostate-tissue samples containing tumor were collected from the patients with localized disease following radical prostatectomy and were embedded in freezing media (Tissue-Tek

OCT Compound, Sakura Finetek, Torrance, CA) and stored in liquid nitrogen. For nine patients from whom LocDCs were collected, the entire prostate was histologically evaluated (see Supplemental Table 1 for matched pairs). The area selected for molecular analysis was from the tumor, which was not multifocal in any of the cases. Tumor cells were isolated by laser-capture microdissection (LCM) from 5-μm thick tissue sections as described previously (20). Two to four thousand cells were collected from each patient and from the normal stroma of five patients. Pathologist (L.D.T.) review of all LCM images confirmed collection of tumor cells. Arrays of stromal-cell samples were used to define thresholds of copy-number change in arrays of primary tumor (see Supplemental Methods and Results).

All other methods are described in Supplemental Methods and Results.

RESULTS

Array CGH can be made compatible with small numbers of cells. To analyze samples containing only 10-20 cells by array CGH, we first amplified the genomic DNA using a ligation-mediated PCR method. This approach, which we term rare-cell genomic amplification (RCGA), was developed by Klein and coworkers to analyze the genome of a single cell by conventional CGH (18). Our CGH platform was a bacterial artificial chromosome (BAC) array, which contains ~4200 BAC clones, each with a known location in the publicly available assembly of the human genome (Build 35), and provides a resolution of ~0.4 Mbp across the euchromatic portions of the genome (21). For all small-cell-number arrays, the reference is normal female genomic DNA amplified by RCGA from an amount of DNA roughly equivalent to the amount in the test sample.

We conducted tests to establish that RCGA and array CGH when applied to small numbers of cells give (1) consistently low levels of experimental noise, (2) acceptable dynamic

range for detecting chromosomal alterations, and (3) reproducibility across replicates. First, we amplified nine samples each composed of 10-20 normal CD45-positive bone marrow-derived cells (from patients with localized prostate cancer) by RCGA and analyzed them by array CGH. We used these normal-cell array results to define thresholds for calling loss and gain (see Supplementary Methods). Applying these thresholds back to the normal-cell arrays, we observed the expected X- and Y-chromosome shifts for all these comparisons of male test to female reference DNA. On average, only 1% (40/4096) and 0.6% (23/4096) of autosomal BACs were encompassed by segments designated as lost and gained, respectively. No site was called a deviation in more than two normal-cell arrays, and only four loci encompassing a total of 28 BACs were called deviant in two of the nine (~20%) normal-cell arrays. Thus, we report below only deviations detected in >20% of LocDC or AdvDC samples in order to minimize the risk of reporting false-positive deviant loci.

We next tested the ability of RCGA and array CGH to detect deviations in small numbers of cells of the LNCaP prostate-cancer cell line, which has well characterized genomic abnormalities (22). Four samples each composed of 10 or 20 cells collected by micropipetting, and one sample comprised of 200 ng of bulk genomic DNA purified from cells of the same passage, were independently amplified by RCGA for array CGH analysis. The analysis of bulk LNCaP DNA identified 13 sites of deviation, all previously reported for this cell line (22). Seven of the 13 (54%) deviant loci observed in the bulk sample were identified in all of the samples of 10-20 cells, and 11 of 13 (85%) were observed in at least one of the arrays from the 10- or 20-cell pools. The two deviations observed in the bulk sample but not in the small-cell-number samples each encompassed only two BACs. Thus, short deviations might be missed in arrays of DNA amplified from a small number of cells.

We found 8.5 additional deviant loci, on average, per array of 10-20 LNCaP cells. We selected five of these deviations to verify by FISH, using one of the BACs encompassed by each deviation as FISH probe. FISH demonstrated a heterogeneous mix of cells with and with-

out the deviation of interest for three of the five copy-number alterations assessed (data not shown). Thus, random selection from a heterogeneous population contributes to the deviations detected by array CGH in small samples of LNCaP cells.

We next demonstrated the reproducibility of our approach using three biological replicates composed of 20 DTCs each collected from a single patient with metastatic prostate cancer. Each 20-cell sample was independently isolated from bone marrow, amplified, labeled, and analyzed by array CGH. Figure 1A shows the high correlation between the normalized $\log_2$-ratio values of each possible pair of replicates. Numerous sites of copy-number change spanning many Mbp of genomic material were identified for this patient's samples. Figure 1B shows the considerable number of consistent deviant segments for the three replicates. Of the 3,077 BACs encompassed by alterations in one or more of these arrays, 72% (2219/3077) were identified in all three cell samples, and 88% (2716/3077) were deviant in two or more samples.

The numerous genomic alterations in AdvDCs include those associated with prostate cancer and disease progression. High-level amplifications as well as loss or gain of many segments encompassing several Mbp were detected in all 11 AdvDC samples. On average, each AdvDC array detected 13 gains and 14 losses. All chromosomes showed a deviation in at least one of the AdvDC samples. Figure 2 summarizes the deviations observed in the AdvDC samples. The list of copy-number changes observed in three or more samples (≥27%) is extensive (Supplemental Table 2).

The minimally overlapping regions (MORs) of copy-number change for deviations observed in at least four samples (36%) are given in Table 1. This list of MORs includes many chromosomal locations reported in the literature as frequent alterations for primary or metastatic prostate tumors, including loss on 8p, 10q, 13q, and 16q, and gain on 8q and Xq. Our results help narrow in on the regions of interest for these prostate-cancer related alterations. We show five discrete MORs on 10q, three on 13q, three on 16q, and four on Xq.

Genomic alterations support the tumor origin of LocDCs. Based on the lack of clear associations between the mere detection of DTCs in men with clinically localized prostate cancer and subsequent relapse, an important question is if what we call DTCs are in fact tumor cells. To address this question, we compared the LocDC array results with the results from the primary tumor and AdvDC arrays. First, we found that the $\log_2$ ratios of the deviant autosomal segments for the LocDCs have a significantly lower dynamic range than those of the primary tumors and AdvDCs (Student's t-test $p < 0.05$). The median $\log_2$ ratio of gained segments in LocDCs was 0.13 (SD = 0.07), in primary tumors 0.21 (SD = 0.11), and in AdvDCs 0.15 (SD = 0.15). The median $\log_2$ ratio of segments classified as loss for LocDCs was -0.15 (SD = 0.10), for primary tumors -0.17 (SD = 0.09), and for AdvDCs -0.17 (SD = 0.15). Second, we found fewer deviations, on average, in LocDCs (avg. = 14) than in primary tumors (avg. = 43) or AdvDCs (avg. = 27).

We determined that normal-cell contamination is unlikely to be responsible for the diminished dynamic range in LocDC arrays. The normalized $\log_2$-ratio values for LocDCs collected using a marker (CD45) to exclude normal hematopoietic cells were not significantly different than those from LocDCs collected without this marker ($p=0.1362$). Moreover, AdvDCs were collected by the same methods as LocDCs, yet showed no evidence of normal-cell contamination.

Despite the less striking amplitude and number of deviations in LocDCs, we provide evidence that they are tumor cells. We exclude experimental noise as being responsible for the deviations observed in LocDCs. We compared LocDC array results to the normal-cell arrays (produced from the nine samples of 10-20 normal CD45+ cells collected and amplified in parallel with the LocDC samples). The deviant segment values for gains and losses of autosomal material in the LocDC and normal-cell arrays were not significantly different (Student's t-test $p = 0.16$ and 0.09, respectively). However, the number of alterations and the amount of genomic

material altered were significantly greater in LocDC arrays than those found in the arrays of 10-20 normal cells. LocDC samples showed an average of 14 deviations (3.5-fold more than normal cells, $p = 0.005$) and ~320 Mbp of altered genomic material (7.8-fold more than normal cells, $p = 0.039$).

Seventeen loci showed copy-number deviation in >20% of LocDCs (Table 2, Figure 3). Thirteen of these loci are likely to be real chromosomal deviations because they have no overlap with deviant segments in normal-cell arrays. Losses involving 13q22 and 19q12 seen in >20% of the LocDCs encompass significantly more BACs than overlapping deviations registered in the normal-cell arrays (18 vs. 9, and 9 vs. 2 BACs respectively) and are thus also candidate tumor-related changes. We discount two deviations (gain in 4p16 and 11q13) as probable experimental artifact, because they appeared in ~20% of normal-cell arrays.

A comparison of the genomic profiles of LocDC and primary tumors indicates several overlapping regions of deviation (Figure 3A and Table 2) supporting their common origin. Nine of the 15 (60%) sites of genomic change detected in >20% of the LocDC samples (excluding the two sites discounted as possible artifact) were also detected as deviant, in the same direction, in >20% of the primary tumor arrays. Moreover, 12 of the 15 loci showing change in >20% of the LocDC samples correspond to previously reported deviations in prostate cancer (Table 2, (23-28)), providing additional evidence that the LocDCs are prostate-tumor cells.

Direct comparison of concordant sites in the nine matched samples of LocDCs and primary tumors provides additional evidence that LocDCs are tumor cells. A concordant deviation was defined as one in which >30% of BACs in one sample's deviation were encompassed by the other sample's deviation, and vice versa. There were a total of 29 concordant sites of deviation across the nine sample pairs (Table 3), an average of 3.2 concordant loci per pair. For 24 of the concordant sites, >50% of BACs were shared. Only one pair lacked concordant alterations. Using simulated data sets, we found that three of the matched pairs have significantly more

concordant changes than the number expected if the deviant segments were randomly distributed across the two genomes.

The degree of concordance we observe is unlikely to be affected by the different amplification schemes used to amplify LocDCs (RCGA) and primary tumors (WGA2) (see Supplemental Methods and Results for detailed comparisons of RGCA and WGA2). In brief, arrays produced from LNCaP DNA amplified by these two methods showed significant concordance in their sites of genomic change ($p<0.0001$) and no significant difference in the dynamic range of the deviant segments ($p=0.4820$).

Frequent LocDC alterations are often detected in AdvDCs but not vice versa. We compared the array-CGH profiles of LocDCs and AdvDCs to look for changes that might illuminate the shared or divergent biology of these cell types. Ten (67%) of the 15 deviations observed in >20% of the LocDC samples (excluding those discounted as artifacts) were also observed in >20% of the AdvDC samples (Table 2). This set of loci includes loss at 8p23, one of the putative tumor suppressor containing regions on 8p (29), which was observed in 27% (3/11) of AdvDC samples and 23% (11/48) of LocDC samples. Two of the four most prevalent changes seen in the AdvDC samples were seen in >20% of the LocDC samples. Loss at 10q26 was observed in 55% (6/11) of AdvDC samples and 25% (12/48) of LocDC samples. Loss at 16q21 was also observed in 55% (5/11) of AdvDC samples and in 31% (15/48) of the LocDC samples.

Given the remarkable degree of genomic change in AdvDCs and the more limited deviations in LocDCs, it is not surprising that the majority of deviations that we observed in >20% of AdvDC samples were deviant in fewer than 20% of LocDC samples (Figure 3B and Supplemental Table 2). For example, losses that overlap with 8p12-21 and 8p22 were observed in four of the eleven (36%) AdvDC samples, five of the nine (56%) primary tumor samples, but only one of the 48 (2%) LocDC samples. Two deviations with considerable frequency differences was a

gain of most of 8q (36% of AdvDCs and 10% of LocDCs) and gain of 1q32 (45% of AdvDCs and 8% of LocDCs).

Regions of frequent chromosomal alteration in DTCs and primary tumors are enriched for genes of involved in specific biological processes. We sought to determine if the chromosomal regions altered in DTCs and primary tumors impact genes of particular biological processes, based on gene ontology (GO) enrichment analysis. We considered all regions altered in >20% of the LocDC, AdvDC, or primary tumor samples (Supplemental Table 3), identified the genes corresponding to sites of loss or gain, and used hypergeometric tests (30) to determine if any GO categories were enriched (at a significance level of $p < 0.001$) in those gene sets. The enriched categories are given in Supplemental Table 4 (losses) and Table 5 (gains). We also include a second p-value, the SimPValue, which is an indicator of those GO categories that might be enriched due to gene clustering. Thus, categories with a SimPValue > 0.05 should be considered with caution.

The notable GO categories significantly enriched in alterations for >20% of the LocDC, AdvDC, or primary tumor samples with a SimPValue < 0.05 are as follows. Of the four categories enriched in the LocDC regions of gain, one was vitamin transport. This particular GO enrichment included FOLR1, a gene whose transcript and protein product are increased in specific epithelial tumors and are positively associated with tumor stage and grade (31). DNA metabolic process and chromatin modification were two of the six categories enriched in primary tumor gains, and cell-cell signaling was one of the 12 categories enriched in primary tumors losses. Various immune related processes made up the six categories enriched in the AdvDC regions of gain. Only electron transport was an enriched category in AdvDC losses (SimPValue = 0.001). One of the electron transport genes in AdvDC regions of loss was the tumor suppressor WWOX (32).

A few enriched gene categories with SimPValue > 0.05 represent biological processes that are sufficiently compelling to note here. Two categories related to adhesion (cell-cell adhesion (SimPValue = 0.051) and homophilic cell adhesion (SimPValue = 0.054)) were enriched in LocDC regions of loss. Transcription, DNA packaging, chromatin assembly, and protein-DNA complex assembly were GO categories enriched in the primary tumor gains. Eight categories related to regulation of transcription, metabolic, cellular, and biological processes were enriched in AdvDC losses.

DISCUSSION

To the best of our knowledge, this study represents the highest resolution genome-wide analysis of changes performed directly on prostate-cancer DTCs. The genome analysis of a small number of cells was made possible by combining RCGA, a whole-genome amplification scheme developed by Klein et al. (18), and array profiling by CGH. The use of a spotted DNA array as a CGH platform detects loss and gain at a high resolution and provides a direct link to the sequence of the human genome, a considerable improvement over conventional methods. Our proof-of-principle studies show that it is possible to produce genomic profiles of a small number of cells that exhibit relatively low levels of experimental noise, highly concordant results between bulk DNA and samples of a small number of cells, and excellent reproducibility across biological replicates.

We demonstrate that LocDCs have real genomic deviations and are composed of tumor cells, even though the genomic deviations in the LocDC samples were generally fewer in number and showed lower dynamic range than those in primary tumors and AdvDCs. First, we see significantly more deviations in the LocDC arrays than in the arrays of a comparably small number of normal cells, arguing against experimental noise as the source of alterations in LocDCs. Second, 80% of the deviant sites detected in >20% of the LocDC samples were previously reported in the literature as altered for prostate tumors. Third, we show significant concordance

between a third of the matched pairs of LocDCs and primary tumors and that 60% (9/15) of the deviations observed in >20% of the all the LocDC samples were identified in our primary tumor samples.

AdvDCs are unmistakably tumor cells, given their numerous genomic alterations. Moreover, AdvDCs frequently possess deviations known to associate with a progressed disease state; deviations that were infrequently observed in LocDCs. A notable example is gain of 8q (36% of AdvDCs and 10% of LocDCs), an alteration associated with advanced disease and poor outcome in patients with organ-confined disease (23, 33, 34). Regions containing the metastasis-suppressor genes MKK4 (17p12) and KAI1 (11p11.2) were each deleted in ~30% of AdvDC samples but very few of the LocDCs. One-third of our AdvDC samples and none of the LocDC samples had a high-level amplification of the region on chromosome X encompassing the androgen receptor gene, a well characterized alteration specific to advanced disease (35, 36). Gain of 1q32 was one of the most common alterations found in AdvDCs (45%), uncommon in LocDCs (8%), and absent in our set of primary tumors. Notably, 50% of the metastatic tumors analyzed by array CGH in a parallel study also have a gain of 1q32 (Holcomb et al., in preparation)[19], indicating that this region is likely to be associated with advanced disease. Will the small number of patients with LocDCs possessing metastases-associated alterations represent a significant proportion of progressors? Long-term follow-up studies are underway to answer this question.

Changes that we frequently observed in both AdvDCs and LocDCs might reflect their common origin or shared state of dissemination. Arguing for the latter, both DTC types showed frequent losses in 8p23, 10q, 13q, and 16q, alterations that have been frequently identified in prostate cancer (33). Deviations found in both types of DTCs, but that were absent from our primary tumor samples might be alterations related to dissemination. These sites include losses

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in 10q25.1, 13q22.2-32.1, 13q32-34, and 16q21. Interestingly, the loss in 16q21 encompasses two members of the cadherin family of adhesion genes (CDH8 and CDH11). Disruption of the cadherin pathway is implicated in tumor invasiveness and disease progression for a number of carcinomas (37). Analysis of a much larger set of matched primary and DTC samples will be needed to confirm what alterations are in fact specifically associated with dissemination.

The reduced amplitude of the genomic changes in LocDCs compared to AdvDCs and primary tumors could have several explanations. Copy-number changes can be dampened in the array-CGH readout by normal-cell contamination, abnormal ploidy, and heterogeneity. As we have shown, normal-cell contamination of our DTC samples is unlikely. Extra ploidy is also unlikely to be a factor in the LocDCs, as it is well established that most early prostate cancers, from which the LocDCs are presumably disseminated, are approximately diploid (38).

Genomic heterogeneity of LocDCs, as is seen in prostate tumors (39), is likely to contribute to a reduction in amplitude of LocDC alterations. Moreover, early dissemination might contribute additional heterogeneity to LocDCs relative to what might be found in the tumor itself. Inclusion of cells that migrated before acquiring multiple genome changes in the pool of LocDCs will dilute and reduce the overall number of detected alterations relative to the primary tumor at the time of resection, which we observe. Early dissemination of LocDCs might also explain their notably infrequent possession of 8p12-22 loss, the most common copy-number alteration reported in prostate cancer (35). Loss in this region was seen in only one of our 48 LocDC samples, in contrast with 54% of our primary tumors and 55% of our AdvDC samples.

How do our LocDC findings fit the current models of metastasis? The two predominant models are that (1) rare, highly metastatic cells within the primary tumor give rise to metastases, or (2) particular tumors, not rare cells, are prone to metastasis. In either case, the implication is that cells leave the primary tumor in possession of the molecular alterations necessary for secondary growth and survival. However, it is hard to imagine that the cells that generate secondary disease in patients many years after the removal of their primary tumor possess full metas-

tatic potential (40). Theories of tumor-cell dormancy have been proposed recently (41-43) suggesting that DTCs from primary tumors might represent a dormant population of cells that can, through selection and mutation over time, acquire key molecular changes that will permit metastatic growth (41). Dormancy is supported by our GO analyses, which showed an enrichment of genes related to proliferation in sites of frequent alteration in our primary tumor and AdvDC samples, but not in our LocDCs, indicating that growth is not a prevailing theme in LocDC alterations. Thus, additional genomic hits would seem to be required, if LocDCs are to become overt metastases.

The high fraction of prostate cancer patients with localized disease in whom DTCs can be detected, and the relatively low level of genomic disorder shown here for such cells are persuasive evidence that LocDCs have not, in the majority of cases, reached full metastatic potential. If a subset of these cells can produce clinically significant metastases, then it seems likely, from the large number of studies showing gross chromosomal deviations in metastatic tumors, that these cells must eventually undergo additional genomic events. However, a subset of the genomic abnormalities that we identified in LocDCs may prove to be harbingers of progressive disease. Follow-up clinical studies of the patient population studied here along with additional molecular characterization of LocDCs could provide insight into the metastatic potential of DTCs.

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TABLES

Table 1. Locations of minimally overlapping regions (MORs) of loss (A) and gain (B) detected in at least four of the 11 AdvDC samples.

A.

Chr.	Start (bp)*	End (bp)*	Size (Mbp)	Loss† (%)
4	8096400	9860900	1.76	36
6	75016100	92151400	17.14	36
6	108616100	119669000	11.05	36
6	159454900	161225200	1.77	36
8	3899600	23478900	19.58	36
10	7937400	11961000	4.02	36
10	43603000	44182000	0.58	55
10	78621100	103535200	24.91	36
10	105726300	111829100	6.10	36
10	129427500	135165800	5.74	55
11	12608600	13908400	1.30	36
11	32183800	36906400	4.72	45
11	107883700	127572100	19.69	36
13	49029900	56820600	7.79	45
13	73284100	73672800	0.39	55
13	80203300	93116600	12.91	55
14	69266000	69440800	0.17	36
15	53535600	61214300	7.68	45
16	53822000	54962800	1.14	36
16	60811600	63807800	3.00	45
16	70010000	84688200	14.68	36
21	26441500	26798300	0.36	36

B.

Chr.	Start (bp)	End (bp)	Size (Mbp)	Gain† (%)
1	161466500	164524500	3.06	45
1	198600100	211134100	12.53	45
2	44100	6608800	6.56	36
2	26184700	27484700	1.30	45
2	42391900	43774500	1.38	36
2	49511100	75725900	26.21	36
2	86778900	110050700	23.27	36
3	4330000	11524200	7.19	36
5	561600	4351700	3.79	36
5	150300400	151093500	0.79	36
5	179467100	180610700	1.14	36
6	52696900	53331800	0.63	36
8	57031700	99396100	42.36	36
9	222300	4407700	4.19	36
9	89644000	98144400	8.50	36
9	111819300	112900900	1.08	45
9	127428500	130252600	2.82	45
16	75800	1753000	1.68	36
17	73516500	78200000	4.68	36
X	7483600	24032000	16.55	36
X	66108300	67961300	1.85	45
X	73682500	99853100	26.17	36
X	148896100	153772100	4.88	36

Abbreviations: **Chr**, chromosome.

*The start and end position are based on Build 35 of the human genome sequence assembly and base pair positions have been rounded to the nearest one hundredth.

†The percentage of AdvDC samples that shows the MOR.

Table 2. Losses (A) and gains (B) observed in >20% of LocDC arrays and the percentage of primary-tumor and AdvDC samples showing an overlapping alteration. A range of percentages is given for most deviations, since not all BACs were encompassed by deviation in the same number of samples.

A.

Losses

Chr.	Band	Size (Mb)	Start (bp)*	% of LocDC (n=48)	% of P (n=9)	% of AdvDC (n=11)	Ref. #
5	p15.32-p15.2	5.92	4443000	23	11	0-9	NA
5	p14.3-p14.1	9.86	18961000	21-23	11	0	NA
8	p23.3-p23.2	4.06	1	20-23	56	27-36	(23)
10	q25.1	5.67	106120000	21	0	36	(24, 25)
10	q26.2-q26.3	6.95	128253000	21-25	0-22	45-55	(23)
12	q24.31-q24.33	6.31	123397000	29-35	33	9-18	NA
13	q14.3-q22.2	22.86	52193000	21-31	11-22	36-55	(23)
13	q22.2-q32.1	19.87	75147000	21-44	0	36-55	(23)
13	q32.3-q34	14.02	99244000	21-23	0	27-36	(23)
16	q12.1-q13.5	5.52	49358000	21-23	33	27-36	(25)
16	q21	7.17	57328000	23-31	0-22	27-45	(23)
19	q12-q13.11	4.23	33315000	29-35	56	27	(23)

B.

Gains

Chr.	Band	Size (Mb)	Start (bp)*	% of LocDC (n=48)	% of P (n=9)	% of AdvDC (n=11)	Ref. #
3	p22.1-p21.1	11.25	42513000	21-33	0-56	9-18	(26)
3	q28-q29	5.21	193293000	21-27	0	18	(26, 27)
4**	p16.3-p16.2	3.36	1178100	29	0	0	NA
9	q33.2-34.11	5.00	125337000	23	22-44	36-45	(28)
11†	q12.1-q13.5	18.96	56620000	23	11-77	18-27	(23)

Abbreviations: **Chr**, chromosome; **P**, primary tumor; **Ref #**, reference reporting deviation.

*The start position is based on Build 35 of the human genome sequence assembly and base pair positions have been rounded to the nearest one hundredth.

** Observed in 22% (2/9) of the arrays performed on 20-cell pools of CD45-positive normal bone-marrow cells.

† Observed in 60% of the arrays performed using LCM-collected normal cells.

Table 3. The number of deviations observed in each matching pair of LocDC and primary tumor samples. The sample pairs with a significant number of concordant deviations ($p < 0.05$) are in bold. The column "concordant" gives the number of deviations for which at least 30% of the encompassed BACs were deviant in both the primary tumor and LocDC sample.

	Number of deviations			
Sample Pair	LocDC	Primary Tumor	Concordant	p-value
1	**49**	**29**	**12**	**<0.0001**
2	16	9	1	0.23
3	12	35	2	0.12
4	**30**	**73**	**9**	**0.0003**
5	2	86	1	0.23
6	14	43	1	0.43
7	15	18	0	1.00
8	4	49	1	1.00
9	**8**	**40**	**2**	**0.03**

FIGURE LEGENDS

Figure 1

Reproducibility of array CGH on RCGA-amplified samples from 10-20 cells. Three pools of 20 AdvDCs were collected independently and amplified independently. Arrays for the three biological replicates were run in parallel against the same female reference. **A**. Scatterplots of the normalized (abbreviated "norm.") $\log_2$-ratio values from each possible pair of replicates. The Pearson's correlation coefficient (r) for each pair is given. **B**. The top plot is a whole-genome profile of the normalized $\log_2$-ratios for one of the replicates. The normalized average $\log_2$ ratio for each BAC is plotted on the y-axis and the midpoint of the genomic position (Build 35) of each BAC on the array is plotted on the x-axis. The three bottom graphs are whole-genome plots of deviant segments for each of the three replicates. The log2-ratio value, as determined by CBS, of each segment exceeding chromosome-specific thresholds is plotted on the y-axis, and the genome position of the deviant segment, as defined by the encompassed BACs, is plotted on the x-axis. Chromosome numbers are indicated along the x-axis.

Figure 2

Frequency of deviations in AdvDCs (n = 11). The frequency of AdvDC samples with a deviation (y-axis) is plotted at the genomic position of the midpoint of each BAC encompassed by the deviation. Blue presents the frequency of gains, and red represents the frequency of losses.

Figure 3

Comparison of copy-number changes observed in LocDC samples, matching primary tumors and AdvDC samples. **A.** Percent of deviations observed across the genome in LocDCs (dots) overlaid on the frequencies for primary tumors (bars). **B.** Percent of deviations observed across the genome in LocDCs (dots) overlaid on the frequencies for AdvDCs (bars). Light blue and orange dots in both A and B indicate the percentage of LocDC samples (n = 48) with observed

copy-number change encompassing each BAC on the array. Blue and red bars give the frequency of copy-number change in the nine matching primary tumor samples (A) or in the 11 AdvDC samples (B). Deviation frequencies of zero are not plotted. The x-axis is the midpoint of the genomic position of each BAC on the array, with chromosome numbers indicated. Note that copy-number loss of chromosome X and gain of chromosome Y represent the normal state for comparisons of male test and female reference genomic DNAs.

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Figure 1

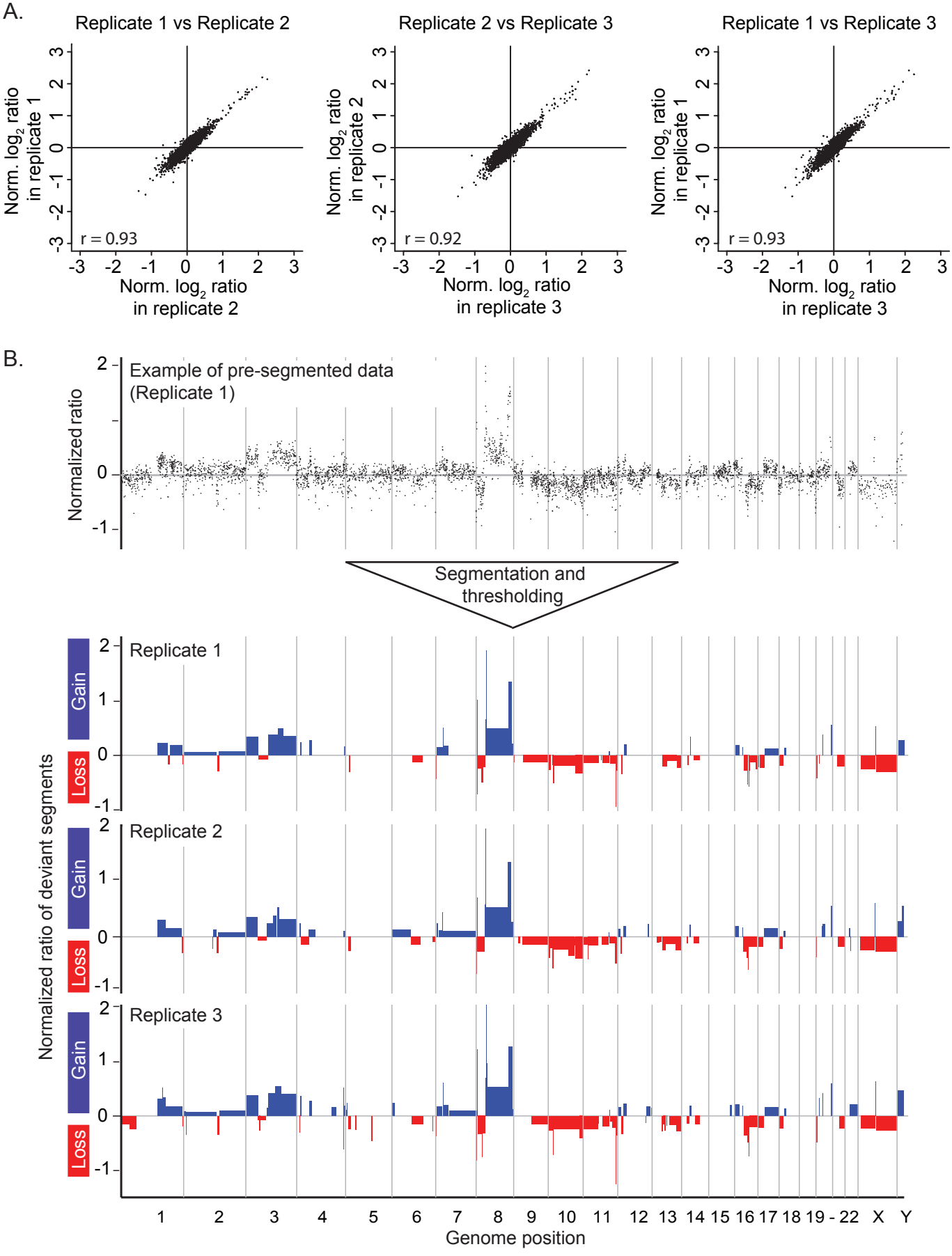

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Figure 2

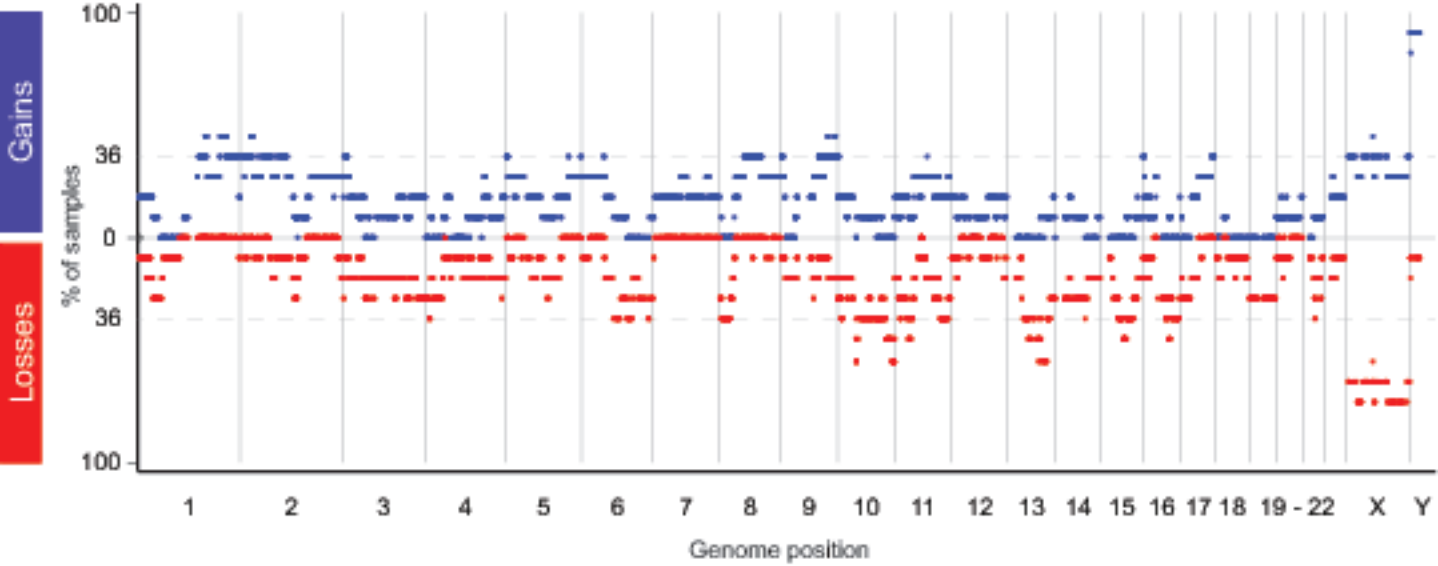

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Figure 3