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Detection of and Isolation of Disseminated Tumor Cells in Bone Marrow of Patients with Clinically Localized Prostate Cancer

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ABSTRACT:

Background: Disseminated tumor cells (DTCs) have been reported in the bone marrow (BM) of patients with localized prostate cancer (PCa). However, the existence of these cells continues to be questioned, and few methods exist for viable DTC isolation. Therefore, we sought to develop novel approaches to identify and, if detected, analyze localized PCa patient DTCs.

Methods: We used FACS to isolate a putative DTC population which was negative for CD45, CD235a, alkaline phosphatase, and CD34, and strongly expressed EPCAM. We

examined tumor cell content by bulk cell RNA sequencing (RNA-Seq) and whole exome sequencing after whole genome amplification. We also enriched for BM DTCs with α-EPCAM immuno-magnetic beads and performed qRT-PCR for PCa markers.

Results: At a threshold of 4 cells per million BM cells, the putative DTC population was present in 10 of 58 patients (17%) with localized PCa, 4 of 8 patients with metastatic PCa of varying disease control, and 1 of 8 patients with no known cancer, and was positively correlated with patients' plasma PSA values. RNA-Seq analysis of the putative DTC population collected from samples above (3 patients) and below (5 patients) the threshold of 4 putative DTCs per million, showed increased expression of PCa marker genes in 4 of 8 patients with localized PCa, but not the one normal donor who had the putative DTC population present. Whole exome sequencing also showed the presence of SNPs and structural variants in genes characteristic of PCa in 2 of 3 localized PCa patients. To examine the likely contaminating cell types, we used a myeloid colony formation assay, differential counts of cell smears, and analysis of the RNA sequencing data using the CIBERSORT algorithm, which most strongly suggested the presence of B cell lineages as a contaminant. Lastly, we used EPCAM enrichment and qRT-PCR for PCa markers to estimate DTC prevalence and found evidence of DTCs in 21 of 44 samples (47%).

Conclusions: These data support the presence of DTCs in the BM of a subset of patients with localized PCa and describe a novel FACS method for isolation and analysis of viable DTCs.

Keywords: metastasis, disseminated cancer cell, dormancy, flow cytometry.

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INTRODUCTION: Prostate cancer (PCa) is the second most common cause of cancer-related deaths of men in the U.S.[1]. Unlike most cancers, late recurrences in PCa are relatively common, with over 20% of recurrences occurring more than five years after curative intent radiation or surgery[2]. These recurrences are thought to result from early dissemination of PCa cells, which initially exhibit a dormant behavior for months or years, but eventually undergo re-activation and lead to clinical recurrence[3,4]. Many of these recurrences may result from distant cells, termed disseminated tumor cells (DTCs) or disseminated cancer cells (DCCs). To find the reservoir for dormancy and subsequent recurrence, investigators have noted PCa avidity for the bone microenvironment, with 90% of fatal metastatic cases involving bone as one of the metastatic sites[5].

Previous investigators have found evidence of DTCs in the bone marrow (BM) from patients with localized PCa using various techniques including; RT-PCR for *KLK3* (PSA), immunocytochemistry for PSA or pan-cytokeratin, and immuno-magnetic enrichment and single cell isolation for EPCAM coupled with immuno-magnetic depletion of normal BM cells,[4,6-24], as we recently reviewed[25]. However, most of these techniques did not allow isolation of viable cells for subsequent mRNA analysis. Furthermore, those that were able to isolate viable cells did not have the advantage of next generation sequencing technologies[4,24]. Most recently, investigators from four institutions were unable to detect DTCs in BM from localized PCa patients using four platforms validated for detection of circulating tumor cells (CTCs) from blood[26].

Therefore, to help reconcile these discrepancies in the literature, we designed novel techniques to detect DTCs in BM of patients with localized PCa, and if present estimate their frequency (*i.e*. number of putative DTCs per million marrow cells) and

prevalence (*i.e.* percentage of localized PCa patients with detectable DTCs). Furthermore, because we ultimately hope to use these techniques to understand the biology of PCa dormancy and recurrence, we desired to isolate viable DTCs so that RNA could be extracted.

Therefore, to detect and isolate viable BM DTCs from localized PCa patients, we designed a fluorescence activated cell sorting (FACS) based protocol and chose high expression of EPCAM as our positive marker, combined with negative markers for possible contaminating BM cell types with no reported expression in PCa cells: CD45 (PTPRC) for hematopoietic cells, CD235a (Glycophorin A) for erythroids, alkaline phosphatase (ALPL) for osteoblastic lineage cells, and CD34 for hematopoietic stem and progenitor cells. As an additional estimate of the prevalence of BM DTCs in localized PCa, we also did a simple immuno-magnetic enrichment for EPCAM and performed qRT-PCR for expression of prostate markers.

Here, using cell enumeration by flow cytometry, bulk cell RNA-Sequencing, whole genome amplification followed by whole exome sequencing, and qRT-PCR, we show evidence for the presence of DTCs in the BM of a subset of patients with localized PCa. These cells are rare, with a frequency less than 1 putative DTC per 10^5 viable, nucleated bone marrow cells in most patients. Our findings are in agreement with the majority of the literature that does find DTCs to be present in some patients with localized PCa, although at a lower prevalence (% of patients) than some reports, which have been as high as 72% of patients[12]. Perhaps more importantly, we have developed methods using standard FACS equipment to isolate viable PCa DTCs. We expect this technical advance to be a boon for investigators studying PCa recurrence.

MATERIALS and METHODS: *Patients and sample collection*: Patient characteristics are summarized in Table I. The use of localized PCa samples for the complimentary approaches is summarized in Figure 1. All subjects provided written informed consent as a part of IRB approved protocols. All patients with localized PCa (AJCC 8th Edition Stage IIIC or less) underwent a radical prostatectomy as their initial treatment of PCa. Any localized PCa patient who received neoadjuvant (before surgery) treatment was excluded from FACS analysis. All samples from patients with localized disease were collected by bone marrow aspiration at Johns Hopkins University either before their surgery from the posterior superior iliac crest, or at the time of surgery from the pubic bone. Samples were transferred to 7.5 ml EDTA (purple cap) tubes and shipped overnight to The University of Michigan on wet ice. Normal marrow samples were purchased from All Cells (Alameda, CA), drawn from the posterior superior iliac crest of paid donors, transferred to heparinized tubes and shipped overnight on wet ice, or aspirated from vertebrae into EDTA tubes during non-cancer spine surgeries at the University of Michigan. Samples from patients with metastatic (all castration resistant) PCa were collected at the University of Michigan by aspiration of the posterior superior iliac crest into EDTA tubes (living donors) or *en bloc* during bisection of the femur or vertebrae at autopsy as part of the Michigan Legacy Tissue Program (rapid autopsy program). Autopsy specimens were homogenized to release cells for analysis.

Anti-EPCAM bead enrichment, mRNA isolation, and qRT-PCR: Enrichment of DTCs from 1 ml of bone marrow aspirate was performed as described for peripheral blood[27]. Briefly, cells were bound to anti-EPCAM magnetic beads, washed, and directly lysed. Messenger RNA was captured with Oligo(dT) 25 mRNA Dynabeads (Thermo

Fisher Scientific) and reverse transcribed into cDNA. Primer sequences were as previously described[27], and all pairs except *KLK2* cross exon boundaries. Preamplification of up to 18 genes including controls was performed followed by qRT-PCR and relative quantification by the ΔΔCt method.

DTC enrichment and/or isolation by FACS: Marrow aspirates were mixed 1:1 with PBS, layered onto Ficoll and centrifuged at 500 x G for 30 minutes to isolate the buffy coat / nucleated population. Subsequent steps were performed in cold flow cytometry buffer (PBS with 2% FCS and 1 mM EDTA). The cells were washed and stained for 1 hour with the following antibodies; PE-Cy7 α-CD235a diluted 1:20 (BioLegend #349112), Brilliant Violet 605 α-CD34 diluted 1:20 (BioLegend #343529), APC α-EPCAM diluted 1:20 (BD #347200), PerCP-Cy5.5 α-alkaline phosphatase diluted 1:20 (BD #561508) and PE α-CD45 diluted 1:5 (BD #555483). Cells were washed and resuspended in flow buffer containing 0.5 µg/ml DAPI and passed through a 40 µm filter to create a single cell suspension for FACS. All analyses were performed on a BD FACS-Aria IIu instrument with 405, 488 and 630 nm lasers. EPCAM positivity was defined as higher than unstained or isotype control stained cells. EPCAM^{high} was defined as at least 5x more intense than the center of the adjacent "dim" population. The putative DTC population was selected from single, viable cells as double negative on a plot of CD45 vs. CD235a, then negative for alkaline phosphatase, then negative for CD34 and with high surface expression of EPCAM on a plot of CD34 vs. EPCAM (Figure 2A). Sorted cells were collected in 10% FCS RPMI in a 0.2 ml tube, washed with cold PBS, leaving behind ~10 µl per tube. 10,000 units of RNAse inhibitor (Invitrogen #55518-012) were added, followed by freezing in liquid nitrogen.

Bulk RNA-Sequencing: FACS isolated marrow samples or 100 C42B cells as a positive control were processed without initial RNA purification using the Takara SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (#634889). cDNA was amplified between 8 and 15 cycles depending on cell number, followed by 16 cycles of library amplification with mRNA optimized methods. 40 million 50-cycle single end reads were obtained on an Illumina HiSeq-4000 instrument. FastQC was used to assess read quality. Data was processed using published methods[28]. Briefly, RNA-Seq reads were aligned to the GRCh38 genome using HiSAT2 (v 2.1.0)[29]. Stringtie (v 1.3.4) was subsequently used to assemble and quantify transcripts from the alignment data[30]. Individual data files were converted to transcripts per million (TPM).

Analysis of gene expression data: Morpheus software (Broad Institute) was used for visualization and hierarchical clustering of gene expression data using default parameters. Gene ontology analysis was conducted with GSEA software (Broad Institute) of the c5.bp.v6.2 gene sets, parameter metric Diff_of_classes, using 5 permutations. The CIBERSORT algorithm[31] (Stanford University) was used to estimate the identity of any immune cells present in RNA-Seq data. Data was uploaded as a text file of TPM values. Data was analyzed using the LM22 (default) signature gene file and the "absolute" mode with 100 permutations.

Whole genome amplification and whole exome sequencing: Samples were processed directly after FACS with no intervening DNA purification. 100 PC3 cells were used as a positive control. Whole genome amplification was performed with the Qiagen single cell Repli-g kit (#150343). Libraries were constructed using an Agilent SureSelectXT Human All Exon V6 kit. Sequencing was performed to ~100x coverage on

an Illumina HiSeq PE150 instrument with 40 million paired end reads per sample. Somatic SNPs were analyzed using MuTect2 software (Broad Institute) tumor vs normal approach with CD45^{+} cells from the same patient as the control for each putative DTC sample. The tumor only approach was used for PC3 cells. Manta software (omicX; Rouen, France) was used for structural variant analysis again using the tumor vs. normal approach except for PC3 cells. We queried a list of the following 75 genes know to be altered in PCa; *PTEN, ZNF292, TP53, FOXA1, ERG, CDKN1B, NEAT1, PDE4D, ROBO2, PPAP2A, ETV3, MLL3, SPOP, MYST3, CDH12, KMT2C, PPP2R2A, ADAM28, IL6ST, UBTF, AR, APC, GPATCH8, ASH1L, DLC1, NCOR2, ZFHX3, TBL1XR1, SENP6, ANTXR2, ARID4B, ASXL2, LCE2B, DOCK10, NDST4, RPL11, RB1, USP28, ARID1A, CASZ1, CNOT3, ATM, PIK3R1, BRCA2, TBX3, ZMYM3, CDK12, KDM6A, NCOR1, CTNNB1, SMAD2, SMAD4, AKT1, BRAF, HRAS, IDH1, KMT2D, MLL2, MTUS1, PIK3CA, PIK3CB, RNF43, FOXP1, SHQ1, RYBP, CDH1, ROBO1, ZBTB16, NCOA7, MYC, MAP3K1, LRP1B, PPE4D, CSMD3, and NKX3-1*[32]. We reported those genes with a SNP or structural variant in the putative DTC sample but not in the internal control.

Cell smear morphologic analysis: Cells were dried on slides, then fixed and stained using the Hema-3 kit (Fisher Scientific). 500 cell counts were performed by a blinded pathologist.

Hematopoietic colony formation: 24 well plates were seeded with 1000 flow events per well and then combined with 500 µl of methylcellulose media with hematopoietic growth factors (Stem Cell Technologies, H4434 Classic #13M53790). Cells were cultured under standard conditions for 7 days without disruption and then

photomicrographed. CFU-E, CFU-GM and CFU-GEMM colonies were counted by a blinded observer.

Statistical analyses: Comparison of 2 groups was performed in GraphPad Prism software by 2 samples unpaired Student's *t*-test. Linear regression was used to assess correlation between putative DTC percentage and patient [PSA]. Histograms were generated using SAS software.

RESULTS: We analyzed bone marrow samples from 72 patients with localized PCa, 8 patients with metastatic prostate cancer (6 living donors, and 2 rapid autopsy patients), and 8 patients with no known cancer as negative controls (6 iliac crest aspirates from paid donors and 2 vertebral marrow samples from non-cancer spine surgeries) (Table I). There was incomplete clinical data for two of the localized PCa patients. The living donor metastatic PCa patients all had treated castration resistant disease and had varying degrees of disease control at the time of donation as approximated by their plasma PSA level. Four were well controlled with PSA values < 6 ng/ml. As described in more detail below, the samples were analyzed using either multi-color FACS followed by downstream analyses on the isolated cells, or enrichment of EPCAM$^+$ cells with immuno-magnetic beads followed by qRT-PCR for PCa markers (Figure 1). To maximize the data collected from these valuable human samples, we split samples between immuno-magnetic bead enrichment (1 ml of marrow aspirate) and FACS analyses (5 – 6 ml) as limited by personnel and equipment availability. Therefore, some patients have data for both the immuno-magnetic bead and FACS based approaches.

Our FACS based method utilized negative markers for erythroid lineage cells (CD235a), hematopoietic stem and progenitor cells and endothelial cells (CD34),

osteoblastic lineage cells (alkaline phosphatase) and set a high threshold for EPCAM

positivity (Figure 2A). To dichotomize the presence of putative DTCs, we set a threshold

of greater than 4 cells per million single, viable, BM cells. This threshold was selected at

a local minimum of a histogram of the frequency of these cells in all samples (Figure 2B,

red line and arrow). Based on the current data, this seemed to be the most reasonable

threshold, but we acknowledge that this threshold line could shift slightly if data from

more patients were acquired. Furthermore, because of the possibility that DTCs were less

numerous than 4 per million, we continued to collect and subsequently analyze the

putative DTC population in samples with lower cell frequencies if at least 20 cells could

be sorted. Because of the rarity of these cells we were limited to collect a maximum of

300 cells from a tube of marrow from localized PCa patients but were able to collect

more from some metastatic patients. Using this threshold of > 4 per million cells, we

detected the presence of the putative DTC population in 4 of 8 metastatic PCa patients,

10 of 58 localized PCa patients, and 1 of 8 normal donors (Figure 2C). In support of a

malignant identity for the putative DTC population, we also noted that the putative DTC

frequency is significantly correlated with localized PCa patients' plasma PSA

concentration; a predictor of recurrence[33]. Clinical recurrence data for this patient cohort

will take years to mature.

In interpreting these results, we note that some of the metastatic PCa patients were

under good disease control (PSA < 6 ng/ml) at the time of sample collection (Table I).

The four metastatic patients with PSA > 6 ng/ml all had a putative DTC population

frequency > 4 cells per million, whereas the metastatic patients with PSA < 6 ng/ml had

DTC population frequencies < 4 cells per million. With regard to the one normal donor

with this population present above threshold, we also note that these methods could theoretically detect most epithelial cancers and at present we are unable to exclude this possibility. Further, marrow from this normal donor subject (AC_6297) demonstrated no molecular evidence of PCa marker gene expression by RNA-sequencing (Figure 3A) or qRT-PCR (Figure 6A) – thus suggesting a false-positive result not due to PCa cells.

To evaluate the tumor identity of the putative DTC population, we analyzed sorted cells from 8 localized PCa patients by bulk RNA-sequencing. Three of these patients (JH3998, JH2695, and JH2517) had a putative DTC frequency above the threshold of 4 cells per million, and five patients (JH3707, JH3756, JH3783, JH3902, and JH4548) were below this FACS threshold. As a comparator for these analyses, we included the putative DTC population from the one normal donor patient that had the putative DTC population present. We also used the C42B cell line as a positive control (Figure 3A). The localized PCa samples are ordered for presentation by decreasing *KLK3* (PSA) expression from left to right. In keeping with the well-established role of PSA as a PCa marker, several other potential PCa marker genes follow the same pattern as PSA. None of the right-most samples (lowest PSA expression) have expression of the PCa marker genes higher than the normal donor sample, with the exception of one sample with *KRT18* expression (patient JH_2695). We interpret this data that four of the eight localized PCa samples had expression of PCa marker genes consistent with the presence of PCa cells. Importantly, the one normal donor sample had no evidence of expression of PCa marker genes. We also analyzed this data set with gene ontology analyses using gene set enrichment analysis (GSEA) methods. Here, we compared the gene expression of the four localized PCa samples with the highest PSA values vs. the one normal donor patient

with this population present. Again, in keeping with the presence of PCa cells in the putative DTC population we noted the highest level of enrichment for the "Cellular Response to Steroid Hormone Stimulus" and "Intracellular Steroid Hormone Receptor Signaling Pathway" gene sets (Figure 3B). We found this to be notable given the importance of testosterone and other steroid hormones in PCa. Conversely, when we compared putative DTC gene expression of the one normal donor to the PCa patients we observed the highest level of enrichment for two immune related gene groups; "Immunoglobulin Production" and "Production of Molecular Mediator of Immune Response," consistent with the presence of marrow cells in these samples.

For further validation of the tumor origin of cells in the putative DTC population, we performed whole genome amplification and whole exome sequencing to ~100x coverage of leukocytes and between 100 and 250 putative DTCs from three localized PCa patients, one metastatic patient as a FACS control, and the PC3 cell line as a sequencing technical control. The metastatic patient (UM_1560) and two of the localized patients (JH_4797, and JH_4905) had > 4 putative DTCs per 10^6 BM cells, and localized patient JH_5557 had 3 putative DTCs per 10^6 BM cells. The leukocyte (CD45$^+$) population was used as an internal germline and technical control for each patient. To determine if these populations contained small alterations characteristic of PCa, we focused our analysis on a list of 75 genes previously reported to be altered in PCa (gene list in methods)[32]. Figure 4A reports genes from this list which had either single nucleotide polymorphisms (SNPs) or gene level structural variants (SVs) in the putative DTC population but not in the corresponding internal leukocyte control. We observed alterations of genes characteristic of PCa in metastatic sample and 2 of the 3 localized

PCa putative DTC samples. Unfortunately, the data across the genome was not

sufficiently uniform to visualize chromosomal level alterations (deletions, amplifications,

and translocations), likely due to unequal amplification, which has been reported

previously with the Repli-G whole genome amplification system[34]. Lastly, to gain an

appreciation for the approximate fraction of cancer cells in the putative DTC population,

we examined the allele frequency for SNPs. The allele frequency was lower for the

localized PCa samples than the metastatic sample, suggesting the continued presence of a

normal marrow population at a higher fraction in the localized samples than the

metastatic sample (Figure 4B). We did not analyze the allele frequency for SVs because

it is more difficult to estimate with the bioinformatics methods used.

    In order to improve isolation methods and to guide selection of any additional

negative markers for future protocol refinement, we further examined the contaminating

marrow cell types in sorted populations (Figure 5). We first performed myeloid colony

formation assays to assess the cell types present (Figure 5A). The putative DTC

population from a localized PCa patient does not yield enough cells for most *in vitro*

assays. Therefore, to achieve a usable number of cells, we decreased the stringency of the

EPCAM gate to yield a population we refer to as "DTC plus," which is like putative

DTCs but also includes EPCAM dim cells in addition to EPCAM high expressing cells.

For comparator cell populations, we also isolated $CD34^+$ cells, $CD45^+$ cells, and "2

marker" cells which were $CD45^-$ / EPCAM $^{dim\ or\ hi}$ but did not include the other negative

markers. The "DTC plus" population showed lower numbers of myeloid colonies than

the "2 marker" population – consistent with contaminating non-myeloid cells in the

putative DTC population. We also analyzed the same populations by morphology on cell

smears (Figure 5B). We observed low numbers of granulocytes in the "DTC plus" population but a higher number of "atypical cells" with an appearance consistent with plasma cells, erythroid precursors or tumor cells. Then, using the RNA-Seq data set first described in Figure 3, we used the CIBERSORT algorithm to estimate the immune cell composition in these samples[31]. The samples are again arranged in decreasing order of PSA expression for the localized PCa samples (Figure 5C). In agreement with the morphological analyses, this highlighted the presence of B-cell sub-sets, especially of plasma cells. We noted a higher estimated content of plasma cells in the samples on the right side of the heat map, which also have lower expression of PCa markers – thus consistent with an increased percentage of plasma cells in samples where the DTC content appears lower. Importantly, the patient with the highest apparent PCa content in Figure 3A (JH_3756) also had the lowest estimated immune cell fraction. Overall, these analyses further support the PCa marker data from Figure 3 and highlight non-myeloid cells, especially plasma cells, as potential contaminating cell types of our putative DTC population.

Lastly, to achieve another estimate of the prevalence of bone marrow DTCs in localized PCa, we used α-EPCAM magnetic bead enrichment and multiplex qRT-PCR for PCa markers to assess the presence of DTCs in 44 localized PCa patients and 4 normal marrow donors (Figure 6). We previously developed these techniques for peripheral blood and reliably detected ≥ 10 PCa cells[27]. In the current work, we observed upregulation of one or more PCa-related genes in approximately half of the localized PCa samples as compared to the normal donor controls (Figure 6A). Importantly, the one normal donor with the putative DTC population present on FACS (AC_6297) was one of

these normal controls and did not show increased PCa marker gene expression. To objectively separate putative DTC positive vs. DTC negative patients we analyzed the localized PCa patient data with hierarchical clustering and observed segregation into three main trunks (Figure 6B). After clustering analysis, we interpreted the outer two trunks as positive – labeled "Positive 1" or "Positive 2" (collectively 21 of 44 samples or 47%) and the center trunk as less likely to contain DTCs – labeled "Negative" or "Equivocal." The six patients labeled "Equivocal" cluster with the "Negative" patients but also share characteristics with "Positive 2." Thus a less conservative interpretation of the data would estimate DTC prevalence at 27 of 44 (61%). We noted that the "Positive 1" trunk was characterized by expression of the pathognomonic PCa fusion gene *TMPRSS2-ERG* and that the "Positive 2" trunk was characterized by expression of *PCA3* and *SCHLAP1*. When the same data was analyzed and presented as the mean gene expression for all patients in each gene expression cluster, we observed increased expression relative to normal donor marrow of 6 of the 8 PCa genes in "Positive 1" and / or "Positive 2" but not in the "Negative" gene expression group (Figure 6C).

Utilization and results from all of the PCa patient and normal donor samples is summarized in Table II. Because the EPCAM bead enrichment and qRT-PCR panel, some patients have PCR data and either RNA-Seq or DNA sequencing data. Not all patients have molecular data available.

DISCUSSION: Here we present multiple lines of evidence indicating BM DTCs are detectable in many men with localized PCa and describe a novel method for their enrichment and collection by FACS – a widely available technique. This study provides important information on the anatomic distribution of PCa cells in clinically localized

disease. At a threshold of 4 putative DTCs per 10^6 BM cells, we conservatively estimate the prevalence of DTCs at 10/58 patients (17%). However, as shown by our RNA-Seq data, some patients with a detectable putative DTC population below this threshold are likely to contain DTCs as well, although their isolation becomes increasingly difficult with current methods. Using qRT-PCR for a panel of PCa marker genes, after EPCAM bead enrichment, we estimate the prevalence of DTCs at 21/44 (47%). Perhaps the real prevalence lies somewhere in the middle. Nevertheless, we feel given the current data it is reasonable to posit that at least some PCa patients do have bone marrow DTCs at the time or radical prostatectomy. We think that these results lend additional insight into the field of PCa dormancy and recurrence, and provide an invaluable tool for investigators studying these processes. These essential techniques could be used in the current form for additional downstream analyses in subsequent work or could serve as the basis for further refinements in DTC isolation.

This work must be placed in the context of prior research on PCa DTCs, as rates of detection have ranged from near zero to 72% of localized patients (reviewed in[25]). Prior to 2018, investigators have reported the presence of DTCs using independent methodologies including; RT-PCR for *KLK3* (PSA), immunocytochemistry for PSA or pan-cytokeratin, and EPCAM based immune-magnetic enrichment and single cell isolation coupled with negative depletion of leukocytes and megakaryocytes. At least one work using each of these techniques has also shown correlation of DTC detection with patient data on recurrence or risk of recurrence.[25] Additionally, our data should be considered in the context of a recent study in which PCa DTCs were very rarely detected at the time of radical prostatectomy (Chalfin *et al*)[26]. It is challenging to prove the

absence of a cell population, as suggested in the study by Chalfin and colleagues, and is also difficult to know the sensitivity of their methodologies. This is particularly true given the use of semi-automated platforms which had been previously validated for the detection of circulating tumor cells (from peripheral blood) rather than for DTCs, and with minimal assessment of positive controls for DTC detection. The unexpected findings of the Chalfin study highlights the need for further confirmatory investigation , such as ours, in part to provide balance. The bulk of the literature supports that BM DTCs are present in some patients with localized PCa – as we have recently reviewed[25]. However, very little is known of the phenotype and behavior of these cells – which will be greatly aided by the techniques for viable cell isolation that we present here. With a few exceptions from groups at the University of Regensburg and University of Washington[4,7,8,18,24], prior investigations of PCa DTCs did not isolate viable cells[6,9-11,13-17,19-23].

Key limitations of the current study include the relatively small sample size and the difficulty isolating sufficient numbers of cells for genetic analyses. Single cell rather than bulk NGS analyses would also be useful but are a significant technical challenge also because of the rarity of the cells. The commonly used single cell platforms from Fluidigm and 10x Genomics require many input cells; on the order of 10^4 or 10^5 – a hundred or thousand fold more DTCs than are likely to exist in a tube of marrow from a localized PCa patient.

An additional limitation of this study is the continued use of EPCAM to help identify DTCs, as EPCAM can lead to both false positives and false negatives. Chery *et al*[4] used single cell micro-array analyses on DTCs isolated using EPCAM based methods

and observed significant EPCAM expression by normal bone marrow cells[4]. Furthermore, DTCs in the bone marrow may downregulate EPCAM as they assume a mesenchymal or stem-like phenotype[35,36]. We acknowledge that some DTCs with a stem or mesenchymal phenotype might not be captured by our current FACS methods. However, we did not think it possible to empirically choose a marker for mesenchymal phenotype DTCs at the onset of this work and hoped to refine our techniques based on data. We think that the data presented here provides an invaluable framework, with which to add additional positive or negative markers in future studies. Our RNA-Seq data is useful this regard. Of the cell surface genes, prostate stem cell antigen (*PSCA*) appears to be highly expressed in DTCs, though analysis of additional patients will be useful. Our work examining the potential contaminating cell types highlights the presence of plasma cells and therefore suggests potential use of plasma cell negative markers. Our data are not inconsistent with the presence of contaminating erythroid precursors as well, as another group previously reported[4]. Together, these data suggest that while normal marrow residents may express EPCAM, higher levels of expression can nevertheless provide valuable information on the presence of DTCs and allow for continued improvement in understanding their biology and how best to isolate them.

CONCLUSIONS: We present multiple lines of evidence demonstrating the presence of bone marrow DTCs in localized PCa patients at the time of radical prostatectomy. Although the fraction of positive patients is perhaps lower than previously reported, DTCs are present in at least some patients at the time of prostatectomy – which provides a clear rationale for further investigation of the bone marrow as a reservoir for PCa recurrence and for the eventual development of therapies targeted to this site. Lastly, we

provide an invaluable tool for viable DTC isolation to better understand the biology of this process – which we hope will lead to fewer cases of deadly recurrent PCa.

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FIGURES

Figure 1: Use of localized PCa samples in complimentary analytic approaches. Fluorescence activated cell sorting (FACS) with multiple markers followed by molecular or morphologic analysis of sorted cells (top), or enrichment with immuno-magnetic beads for EPCAM positive cells and multiplex qRT-PCR (bottom).

Figure 1

Bulk RNA-Seq, $n = 8$

Whole genome amplification and whole exome sequencing, $n = 3$

FACS analysis, $n = 58$

Stained cell smears for morphologic analysis, $n = 5$

72 Localized PCa Samples

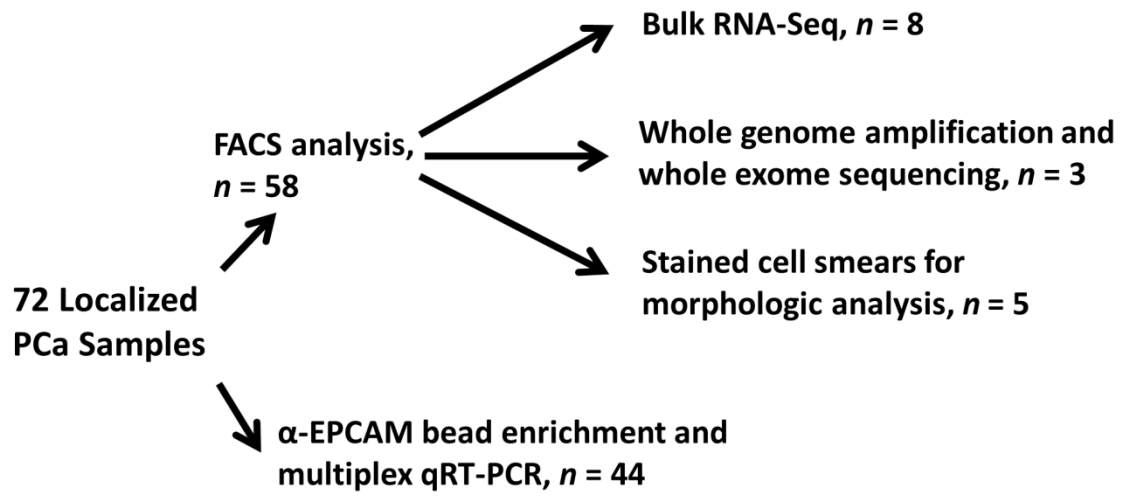α-EPCAM bead enrichment and multiplex qRT-PCR, $n = 44$

Figure 2: Analysis and isolation of putative DTCs using multi-parameter FACS. **A,** Flow cytometry markers and gating strategy. **B,** Histogram of the frequency of putative DTCs per 10^6single, viable, BM cells from all sample types: normal donors; $n = 8$, localized PCa; $n = 58$, and metastatic PCa; $n = 8$. A red threshold line is drawn at a local minimum at 4 cells per 10^6 where no data points were present. **C,** Frequency of the putative DTC population in normal donors, localized PCa patients and metastatic PCa patients. The red dotted line indicates the FACS positive threshold of 4 cells per 10^6. **D,** Correlation of localized PCa patient DTC frequency vs. blood PSA concentration by linear regression analysis.

Figure 2

A

Marker	Target
DAPI	Viable cells
CD45	Leukocytes
CD235a	Erythroid cells
alkaline phosphatase	Osteoblastic lineage
CD34	HSCs and progenitors
EPCAM / CD236	Epithelial cells

FACS gaging of single, viable buffy coat cells

B

Putative DTCs / 10^6 viable cells

C

Putative DTC Frequency

Fraction above threshold:
1/8   10/58   4/8

D

Localized PCa DTC Frequency vs. PSA
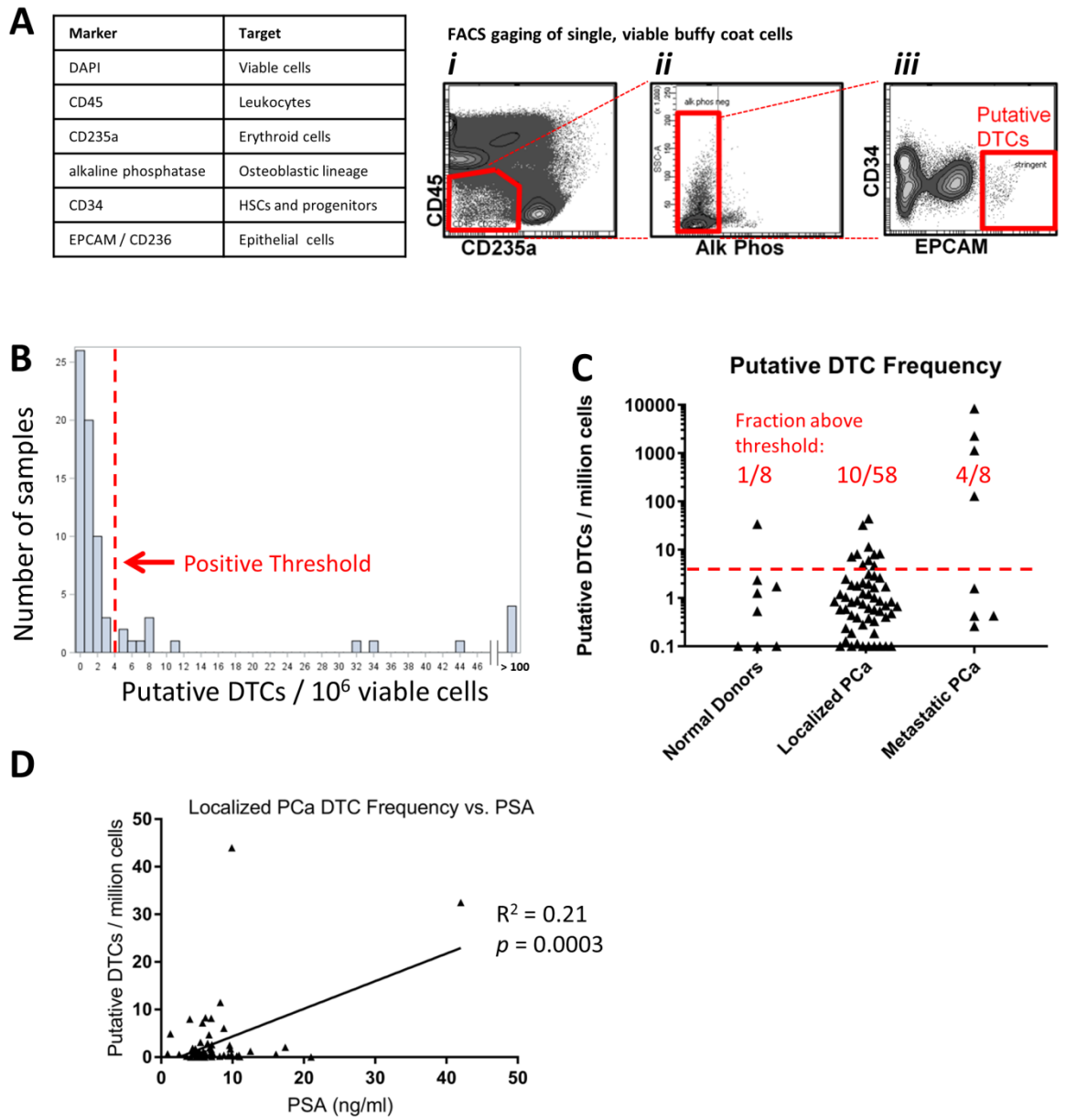
$R^2 = 0.21$
$p = 0.0003$

Figure 3: Examination of BM putative DTC expression profiles by RNA Sequencing. **A**, Heat map of relative TPM values of proposed PCa marker genes from samples of the FACS population containing putative DTCs. Samples are color coded as follows: gray; the one control patient with this population, orange; C42B cell line as a positive control, and blue; 8 Localized PCa patients. The localized PCa samples are ordered from left to right in decreasing order of *KLK3* (PSA) expression. **B,** Gene ontology analysis using GSEA software comparing the 4 highest *KLK3* expressing localized PCa samples relative to the normal donor sample. Top: gene sets with the 10 highest normalized enrichment scores are listed. Bottom: Enrichment plots for the gene sets with the two highest normalized enrichment scores. **C,** Results from the gene ontology analysis shown in (B), for gene sets enriched in the normal donor relative to the localized PCa patient samples.
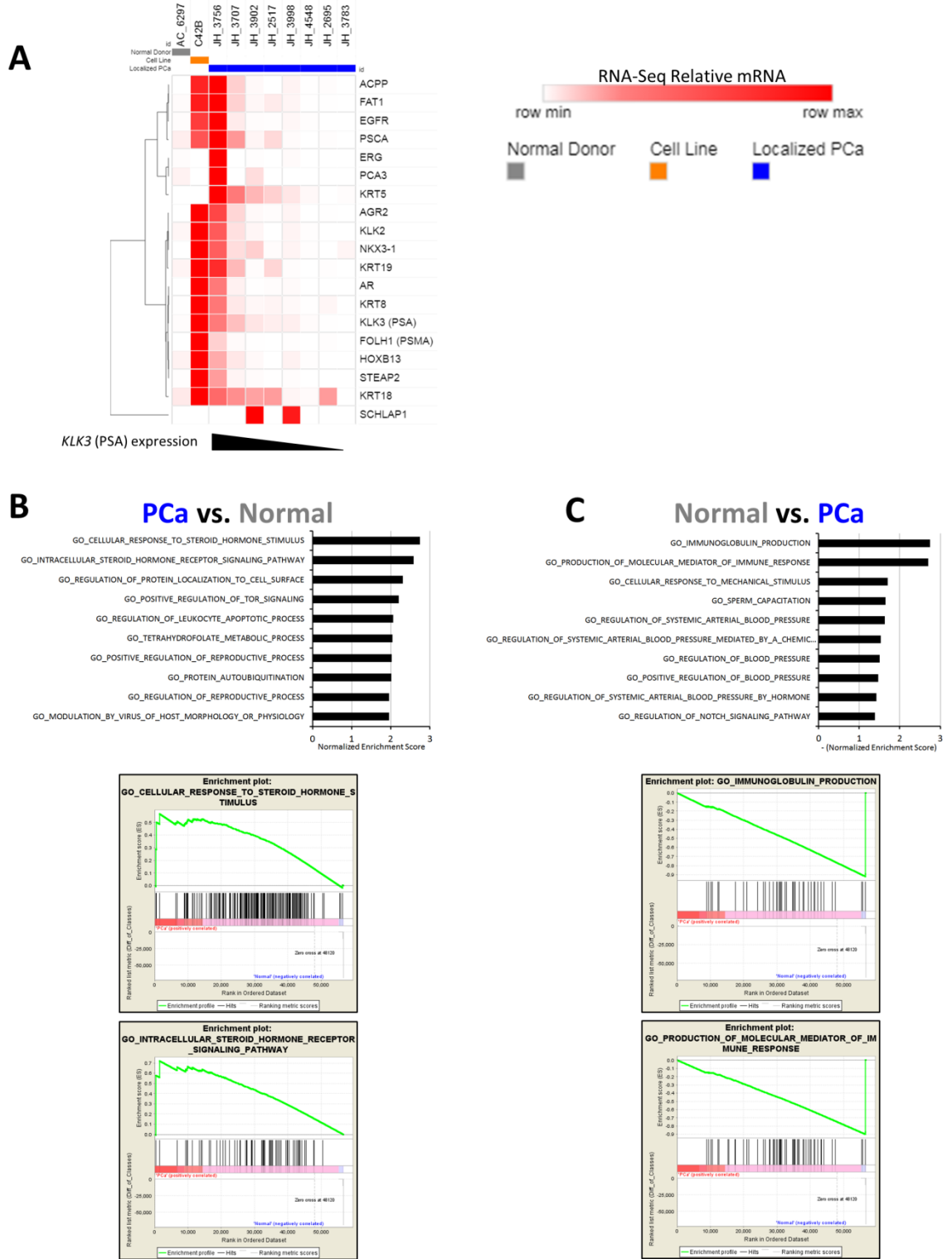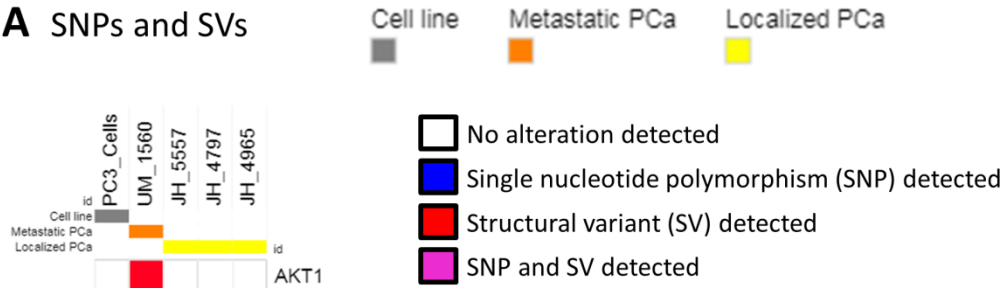
Figure 3

Figure 4: Whole genome amplification and whole exome sequencing of the putative DTC population. **A,** Map showing somatic single nucleotide polymorphisms (SNPs) or small structural variants (SVs), detected in each experimental sample but not CD45^{+} cells from the same patient as an internal control. Sample types are annotated as follows: gray; PC3 PCa cell line, orange; metastatic PCa patient, yellow; 3 localized PCa patients. Detected alterations are indicated by: white; no alteration detected, blue; SNP detected, red; SV detected, purple; SNP and SV both detected. All genes were selected from a list of 75 genes previously reported to be altered in prostate cancer. **B,** Allele frequencies for SNPs detected in the PCa putative DTC samples and PC3 cells. The median allele frequency for each sample is listed in the bottom row.

Figure 4

A SNPs and SVs

Cell line (gray) **Metastatic PCa** (orange) **Localized PCa** (yellow)

- ☐ No alteration detected
- ■ Single nucleotide polymorphism (SNP) detected
- ■ Structural variant (SV) detected
- ■ SNP and SV detected

DNA alterations detected in putative DTCs, relative to internal germline control. From a list of 75 genes commonly altered in PCa

Columns: PC3_Cells, UM_1560, JH_5557, JH_4797, JH_4965

Genes: AKT1, APC, AR, ARID1A, ASH1L, ASXL2, ATM, CDH1, CDK12, DOCK10, ERG, ETV3, FOXA1, FOXP1, IDH1, KAT6A, KMT2B, KMT2C, KMT2D, LCE2B, LRP1B, MAP3K1, MTUS1, NCOA7, NCOR1, RB1, RNF43, ROBO1, TP53, UBTF, ZBTB16, ZFHX3, ZMYM3

B SNP Allele Frequencies

	Cell line	Metastatic PCa	Localized PCa	
	PC3_Cells	UM_1560	JH_5557	JH_4797
AR				0.318
ARID1A	0.114	0.076		
ASH1L	0.135			
CDH1				0.4
CDK12	0.145			
ERG	0.13			
FOXA1	0.771	0.804		
FOXP1	0.126			
IDH1	0.892			
KAT6A		0.06		
KMT2B	0.193			
LCE2B			0.123	
MTUS1		0.904		
NCOA7	0.23			
NCOR1		0.88	0.054	
RB1				0.138
RNF43	0.235			
ROBO1	0.827			
TP53	0.954	0.075	0.066	
UBTF			0.072	
ZFHX3	0.181	0.978	0.919	
Median	**0.193**	**0.804**	**0.098**	**0.318**

Figure 5: Identity of contaminating cell types. **A**, Estimation of the number of contaminating myeloid precursors by colony formation. Upper left; example images of each colony type. Upper right; Example FACS plots describing the cell populations examined. Data are a representative experiment mean ± SD of the six rows of a 24 well plate. Means were compared by Student's *t*-test. **B**, Estimation of contaminating cell types by morphology. Top: Hema3 (Wright-Giemsa) stained smears prepared after sorting the indicated cell populations: Bottom: 500 cell marrow differential cell counts were performed by a blinded observer of smears from five patients. Data represent mean ± SEM. **C**, Estimation of immune cell content in the putative DTC population from the RNA-Seq data (as in Figure 3) using the CIBERSORT algorithm.

Figure 5

Figure 6: Evaluation of localized PCa DTCs by anti-EPCAM bead enrichment and qRT-PCR. **A**, Expression of 8 potential PCa marker genes in bone marrow from 44 patients with localized PCa (light blue) relative to four bone marrow donors with no known cancer (orange). **B**, Hierarchical clustering of marker gene expression in the PCa patients. Of the three main clusters, the outside two clusters are hypothesized to contain DTCs ("Positive 1" (blue) and "Positive 2" (purple)). The middle cluster is hypothesized to be "Negative" (yellow) or "Equivocal" (green) for the presence of DTCs. The three main clusters are indicated by the tree above, and brackets below the heat map. **C**, Mean ± SEM expression of the marker genes grouped by healthy donor or the four PCa patient expression clusters.
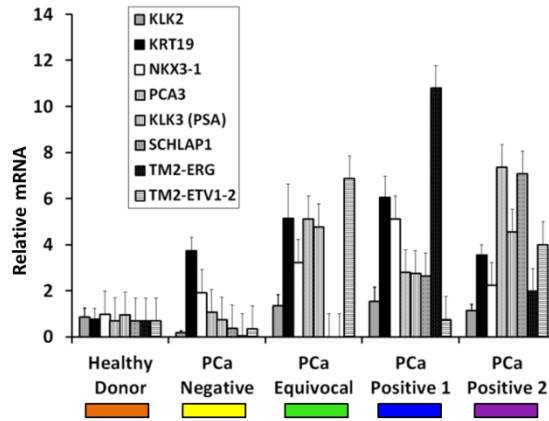
Figure 6

A All Samples, no Clustering
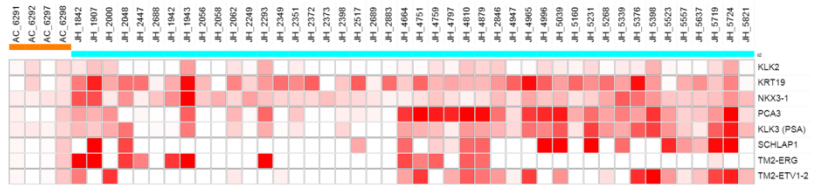
B PCa Samples, with Clustering
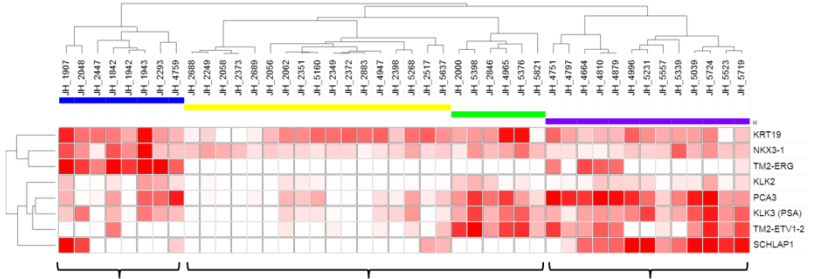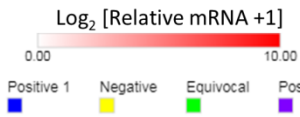
Hierarchical clustering first order grouping: 1   2   3

C Mean PCa Marker Expression by Cluster

Localized Prostate Cancer Patients

	Criterion	Subjects (n)
Total	-	72
Age (years)	≤ 59	19
	60 -69	39
	≥ 70	12
PSA (ng/ml) before prostatectomy	≤ 6	32
	6.01 - 10	28
	10.01 - 20	8
	> 20	2
Prostatectomy Gleason score	6	3
	3+4 = 7	45
	4+3 = 7	12
	8 - 10	10
Tumor stage	pT2	41
	pT3a	23
	pT3b	7
Nodal stage	pN0	59
	pNx	11
Surgical margin	Negative	57
	Positive	13
Collection site	Iliac crest	27
	Pubis	43

Metastatic Prostate Cancer Patients

	Criterion	Subjects (n)
Total	-	8
Age (years)	≤ 59	3
	60 -69	3
	≥ 70	2
PSA (ng/ml) before collection	≤ 6	4
	6.01 - 10	1
	10.01 - 20	0
	> 20	3
Collection site	Iliac crest	6
	Vertebrae	1
	Femur	1

Patients with no Known Cancer

	Criterion	Subjects (n)
Total	-	8
Sex	Male	5
	Female	3
Age (years)	≤ 29	4
	≥ 30	4
Collection site	Iliac crest	6
	Vertebrae	2

Table I: Patient characteristics

Clinical / FACS category	Total	DTCs collected	RNA-Seq positive	RNA-Seq negative	WGA / WES positive	WGA / WES negative	qRT-PCR positive	qRT-PCR negative or equivocal
Normal donor DTC negative	7	0	0	0	0	0	0	3
Normal donor DTC positive	1	1	0	1	0	0	0	1
Localized PCa DTC negative	48	8	3	2	1	1	11	13
Localized PCa DTC positive	10	9	1	2	1	0	1	5
Localized PCa No FACS data	14	0	0	0	0	0	10	4
Metastatic PCa DTC negative	4	0	0	0	0	0	0	0
Metastatic PCa DTC positive	4	4	0	0	1	0	0	0

Table II: Results summary. Left column "Negative" and "Positive" denote either less than 4 putative DTCs per million BM cells or more than 4 putative DTCs per million BM cells respectively. "RNA-Seq positive" denotes increased expression of PCa marker genes on RNA sequencing of putative DTCs. "WGA / WES positive" denotes the presence of mutations in PCa genes after whole genome amplification and whole exome sequencing.