Circulating Fibroblast-Like Cells in Men With Metastatic Prostate Cancer

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BACKGROUND. Metastatic prostate cancer is an incurable disease. During the development of this disease, prostate cancer cells enter the bloodstream as single cells or clusters of cells. Prostate fibroblasts, a cancer-promoting cell type in the prostate cancer microenvironment, could in theory incorporate into these migrating cell clusters or follow cancer cells into the bloodstream through holes in the tumor vasculature. Based on this idea, we hypothesized that fibroblast-like cells, defined here as cytokeratin 8/18/19$^-$/DAPI$^+$/CD45$^-$/vimentin$^+$ cells, are present in the blood of men with metastatic prostate cancer.

METHODS. Veridex's CellSearch® system was used to immunomagnetically capture EpCAM$^+$ cells and clusters of cells heterogeneous for EpCAM expression from the blood of men with metastatic prostate cancer, localized cancer, and no known cancer, and immunostain them for the presence of cytokeratins 8/18/19, a nucleus, CD45, and vimentin. Fibroblast-like cells were then quantified.

RESULTS. Fibroblast-like cells were present in 58.3% of men with metastatic prostate cancer but not in any men with localized prostate cancer or no known cancer. The presence of these cells correlated with certain known indicators of poor prognosis: $\geq$5 circulating tumor cells, defined here as cytokeratin 8/18/19$^+$/DAPI$^+$/CD45$^-$ cells, per 7.5 ml of blood, and a relatively high serum prostate-specific antigen level of $\geq$20 ng/ml.

CONCLUSIONS. The presence of fibroblast-like cells in the blood may provide prognostic information as well as information about the biology of metastatic prostate cancer. *Prostate 73: 176–181, 2013.* © 2012 Wiley Periodicals, Inc.

KEY WORDS: **metastasis; blood; vimentin**

INTRODUCTION

Metastatic prostate cancer poses a significant risk to men, with 67% of men dying within 5 years of diagnosis [1]. This disease is often treated initially with hormone therapies but almost always eventually becomes resistant [2]. In order to develop novel strategies for preventing and/or treating metastatic prostate cancer, we need to better understand the biology of metastasis, including the biology of the blood during metastasis.

Before cancer cells capable of metastasis enter secondary sites, they must first invade through the basement membrane at the primary tumor site and enter

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the bloodstream as single cells or in cell clusters [3]. Migrating clusters of cancer cells could, in theory, incorporate non-cancer cell types such as prostate fibroblasts, a cell type in the prostate cancer microenvironment that induces prostate cancer growth [4–7] and invasion [8]. Lending credence to this theory is the fact that metastatic prostate cancer cells express high levels of *N*-cadherin [9], a protein that mediates their adhesion to prostate fibroblasts [10].

It is also possible that prostate fibroblasts enter the bloodstream as single cells. Tumor vasculature is leaky [11], suggesting that cells from a tumor microenvironment could enter the bloodstream through holes in vessel walls. Furthermore, prostate fibroblasts invade through collagen I in response to prostate cancer-derived angiogenin [12,13], suggesting that they may follow migrating prostate cancer cells into the bloodstream.

Based on these concepts, we hypothesized that fibroblast-like cells are present in the blood of men with metastatic prostate cancer but not in the blood of men with localized prostate cancer or no known cancer. To test this hypothesis, we used Veridex's CellSearch® system to capture circulating $CK^-/DAPI^+/CD45^-/vim^+$, or fibroblast-like, cells, which were then quantified. The presence of fibroblast-like cells in the blood may provide both prognostic information and information about the biology of metastatic prostate cancer.

MATERIALS AND METHODS

Study Participants

Participants with localized prostate cancer and those with metastatic, castration-resistant prostate cancer were identified in a medical oncology clinic, while cancer-free participants were recruited through advertising for normal controls. All participants consented to participate in this study per IRB-approved protocol. Their characteristics are delineated in Table I.

Blood Sample Collection

Blood samples (10 ml/sample) from 14 men with metastatic prostate cancer (2 for an initial experiment, presented in Fig. 1B,C), 10 with localized prostate cancer, and 9 without cancer were collected in CellSave Preservative tubes. During the participant visits when these blood samples were collected, blood samples were also collected for serum PSA measurements.

TABLE I. Medical information of study participants

Cancer status	Participant	Age	PSA level (ng/ml)	Gleason score	Treatment
Localized PCa	10	64	7.9	3 + 3 = 6	None
	11	70	7.1	3 + 4 = 7	None
	12	64	10.3	4 + 3 = 7	None
	13	63	6.4	3 + 3 = 6	None
	14	70	8.4	4 + 3 = 7	None
	15	67	5.9	3 + 4 = 7	None
	16	53	14.0	3 + 4 = 7	None
	17	68	5.6	3 + 4 = 7	None
	18	54	4.1	3 + 4 = 7	None
	19	60	2.2	3 + 3 = 6	None
Metastatic PCa	20	46	4.7	4 + 4 = 8	H
	21	86	160.3	7	RRP C H
	22	72	12.6	4 + 4 = 8	RRP RT H
	23	69	75.3	4 + 3 = 7	RT C H
	24	57	16.4	9	RRP H
	25	82	176.2	3 + 3 = 6	RT C H
	26	65	124.2	3 + 4 = 7	C H
	27	54	4.9	4 + 5 = 9	H
	28	66	95.3	4 + 3 = 7	RRP RT C H
	29	80	68.5	5 + 4 = 9	RRP C H
	30	62	7.7	7	RRP H
	31	76	582.0	7	RT C H

Participants 1–9 had no known cancer, and their ages ranged from 22 to 60.
RRP, radical retropubic prostatectomy; RT, radiation therapy at the primary tumor site; C, chemotherapy; H, hormone therapy.

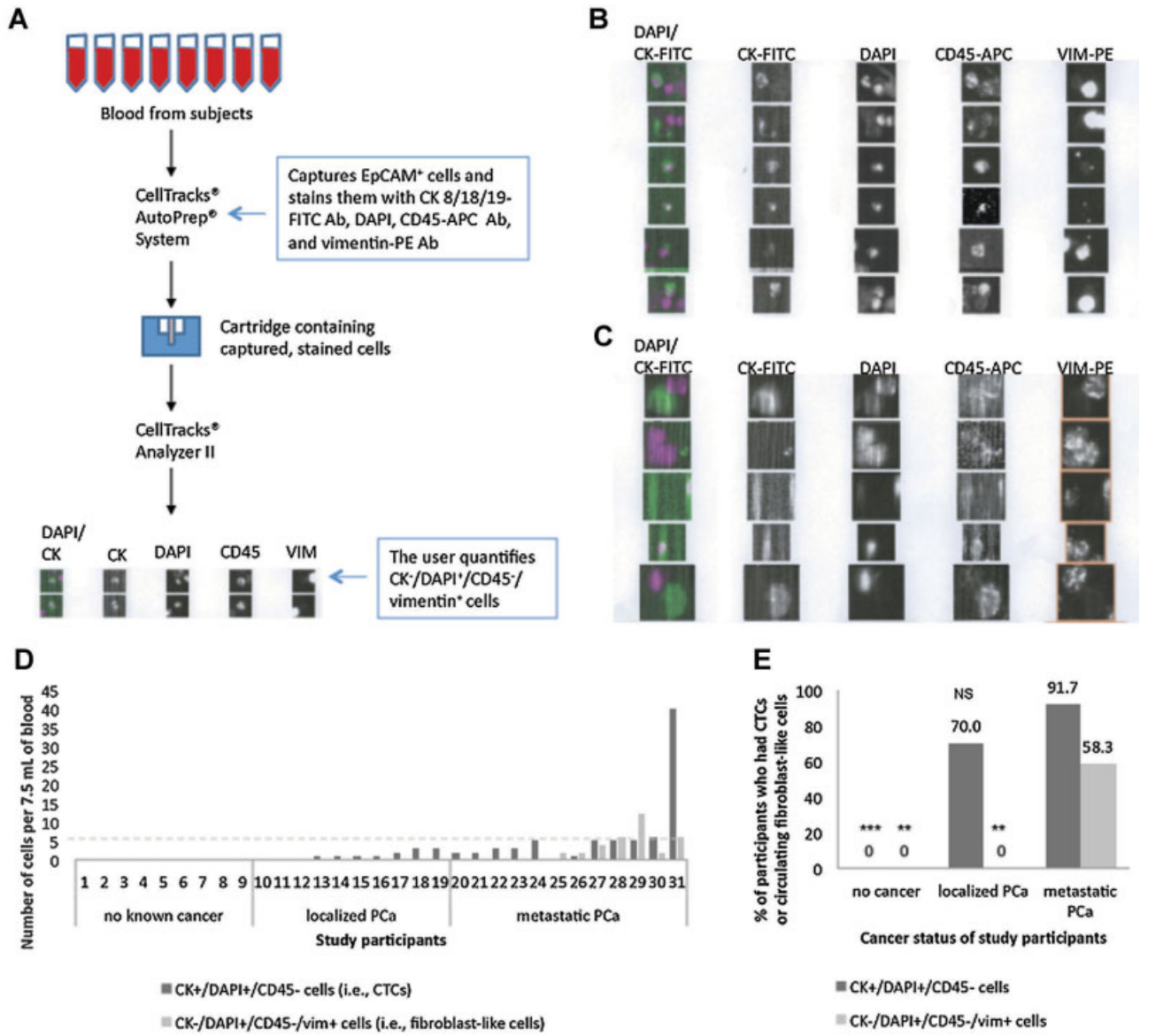

Fig. 1. CellSearch® system protocol and resultant images and cell counts. **A**: Diagram depicting the CellSearch® system protocol. Blood was collected from men with no known cancer, localized prostate cancer, and metastatic prostate cancer, and loaded onto a CellTracks® AutoPrep® system. This system captured EpCAM$^+$ cells, and clusters of cells heterogeneous for EpCAM expression, and stained them for the epithelial markers cytokeratins 8/18/19 (CK); the presence of a nucleus (DAPI); the lymphocyte marker CD45; and the mesenchymal marker vimentin (vim). The captured, stained cells were transferred to a CellTracks® Analyzer II and scanned into CellSearch® software, which presented the user with images of cells that could then be quantified. All of the images in a single row depict the same cell or cell cluster. **B**,**C**: Subsets of images of captured, stained cells from one study participant (B) and a second study participant (C) who had metastatic prostate cancer. **D**: The number of CK$^+$/DAPI$^+$/CD45$^-$ cells (i.e., circulating tumor cells, or CTCs) and CK$^-$/DAPI$^+$/CD45$^-$/vim$^+$ cells (i.e., circulating fibroblast-like cells) per 7.5 ml of blood in 9 men with no known cancer, 10 with localized prostate cancer (PCa), and 12 with metastatic PCa. **E**: The percentage of study participants in each category (e.g., no known cancer, localized PCa, and metastatic PCa) who had CTCs or circulating fibroblast-like cells. ***, $P < 0.001$; NS, $P > 0.05$, compared to CK$^+$/DAPI$^+$/CD45$^-$ cells in participants with metastatic PCa. **, $P < 0.01$, compared to CK$^-$/DAPI$^+$/CD45$^-$/vim$^+$ cells in participants with metastatic PCa.

All blood samples were collected under IRB-approved protocols at the Johns Hopkins University School of Medicine (JHSOM) and the University of Michigan Medical School. The CellSave tubes were gently inverted five times and then stored at room temperature for up to 72 hr prior to blood sample processing as described in Veridex's CXC kit protocol.

Immunomagnetic Isolation of Cells

Immediately after processing, the blood samples (7.5 ml/sample) were loaded onto a CellTracks® AutoPrep® System (Fig. 1A), where they were immunomagnetically enriched for $EpCAM^+$ cells and clusters of cells heterogeneous for EpCAM expression. The system stained the enriched cells with the CXC kit reagents DAPI, FITC-labeled anti-CK-8/18/19 antibody, and APC-labeled anti-CD45 antibody, as well as the user-defined reagent PE-labeled anti-vimentin antibody (Abcam, diluted to 4 or 2 μg/ml in 0.1% bovine serum albumin/PBS for Fig. 1B,C, respectively, and 3 μg/ml in 0.1% bovine serum albumin/PBS for Figs. 1D,E and 2). A cartridge containing the enriched, stained cells was removed from the system and placed in the dark for 20 min to 24 hr prior to analysis.

Analysis of Captured Images

The cartridge containing the enriched, stained cells was placed in the CellTracks® Analyzer II, where the cells were scanned (0.5 sec for the PE channel). Images of single cells and small cell clusters were then loaded into the CellSearch® software and reviewed by multiple individuals, who enumerated $CK^-/DAPI^+/CD45^-/vimentin^+$/cells (i.e., circulating fibroblast-like cells) and $CK^+/DAPI^+/CD45^-$ cells (i.e., circulating tumor cells).

Statistics

For the data presented in Figures 1E and 2A–C, Fisher's exact test was performed using a Microsoft Research online calculator, which generated two-sided *P* values.

RESULTS

The overall goal of this study was to determine whether circulating $CK^-/DAPI^+/CD45^-/vimentin^+$, or fibroblast-like, cells are present in men with metastatic prostate cancer. In a preliminary study (outlined in Fig. 1A), we found that these cells were indeed present in both men that we tested; images of fibroblast-like cells from these men are shown in Figure 1B,C. These results led us to question whether the presence of circulating fibroblast-like cells is a specific feature of metastatic prostate cancer or is generalizable to all prostate cancers or even all men. Thus, we performed a larger study in which we evaluated their presence in 12 men with metastatic prostate cancer, 10 with localized prostate cancer, and 9 with no known cancer (Table I contains relevant medical information of these participants). Circulating fibroblast-like cells were detected in 7 of 12 men with metastatic prostate cancer and in none of the men with localized prostate cancer or no known cancer (Fig. 1D,E).

In men who had detectable levels of circulating fibroblast-like cells, the cell number ranged from 2 to 12 per 7.5 ml of blood (Fig. 1D). The presence of these cells correlated with certain known indicators of poor prognosis: ≥5 circulating tumor cells (CTCs, or $CK^+/DAPI^+/CD45^-$ cells) per 7.5 ml of blood, and a relatively high serum prostate-specific antigen (PSA) level of ≥20 ng/ml (Fig. 2).

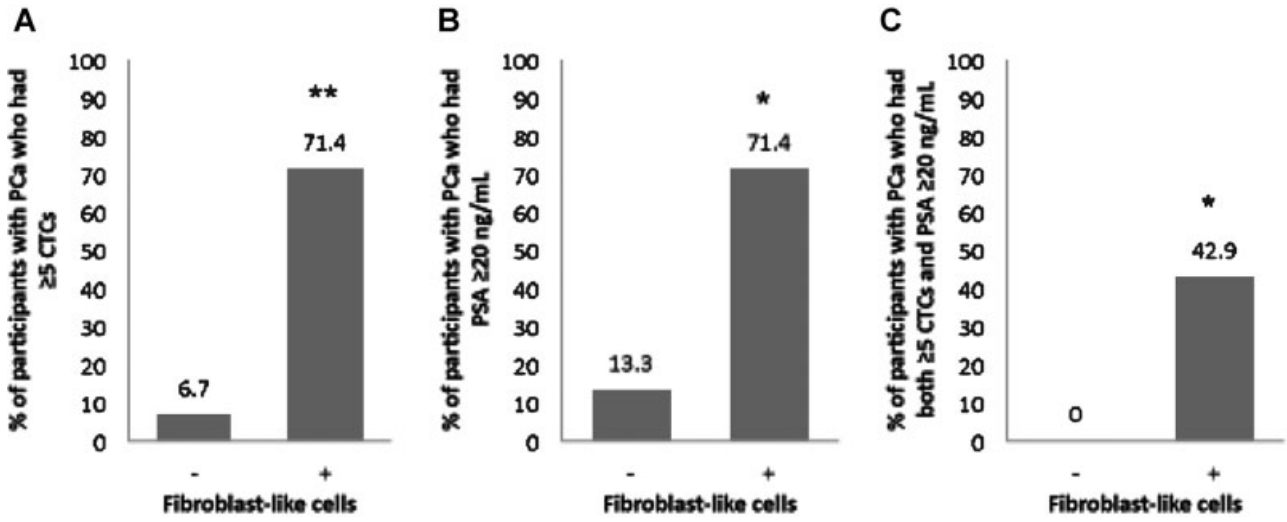

Fig. 2. Correlation of the presence of circulating fibroblast-like cells with known indicators of unfavorable prostate cancer prognosis: ≥5 CTCs per 7.5 ml of blood (**A**), a PSA level of ≥20 ng/ml (**B**), and both ≥5 CTCs per 7.5 ml of blood and a PSA level of ≥20 ng/ml (**C**). **, $P < 0.01$; *, $P < 0.05$, compared to no circulating fibroblast-like cells.

DISCUSSION

We found that circulating $CK^-/DAPI^+/CD45^-/vimentin^+$, or fibroblast-like, cells were indeed present in study participants with metastatic prostate cancer but not in those with localized prostate cancer or no known cancer. While others have found circulating cells expressing mesenchymal markers in men with prostate cancer [14], those cells also expressed epithelial markers such as cytokeratins. In contrast, we detected circulating cells that express the mesenchymal marker vimentin but not cytokeratins. To our knowledge, we are the first to detect such cells in prostate cancer patients. This finding may have both clinical and biological importance.

From a clinical standpoint, the presence of circulating fibroblast-like cells in men with prostate cancer may indicate a poor prognosis. These cells were detected only in men with metastatic prostate cancer, an incurable disease. Furthermore, the presence of these cells correlated with certain known indicators of poor prognosis: $\geq$5 circulating tumor cells (CTCs, or $CK^+/DAPI^+/CD45^-$ cells) per 7.5 ml of blood, and a relatively high serum prostate-specific antigen (PSA) level of $\geq$20 ng/ml (Fig. 2). These cut-off values for CTCs and PSA are controversial: depending on the source, $\geq$4 [15] or $\geq$5 CTCs [16] per 7.5 ml of blood, or a post-prostatectomy PSA level of $\geq$0.2 ng/ml [17] or $\geq$0.4 ng/ml [18], to name just two, indicates poor prognosis. Circulating fibroblast-like cells differ from CTCs and PSA in that it is their presence rather than any arbitrary cut-off value that seems to correlate with poor prognosis. Thus, these cells may be more meaningful than CTCs and PSA as a prognostic indicator.

From a biological standpoint, our work raises several important questions about the pathology of metastatic prostate cancer. First, did the fibroblast-like cells that we detected originate from the prostate? If so, were they prostate fibroblasts, prostate myofibroblasts (i.e., cells having characteristics of both smooth muscle cells and fibroblasts), or prostate cancer cells that underwent epithelial-to-mesenchymal transition? We are working on characterizing the fibroblast-like cells to address these questions.

Another important question raised by our work is where the circulating fibroblast-like cells go, and more specifically, whether they go to the bone, a predominant site for metastatic prostate cancer. The current dogma is that the stromal cells, that is, non-epithelial cells, in the bone of men with metastatic prostate cancer are bone-derived [19]. Our findings give rise to the possibility that some of the stromal cells in their bones are prostate-derived: if the circulating fibroblast-like cells that we found are indeed prostate fibroblasts, then perhaps prostate fibroblasts migrate to secondary sites such as the bone and help cancer grow at those sites. In support of this hypothesis, Duda et al. [20] found in a mouse model that carcinoma-associated fibroblasts migrated with metastasizing cancer cells to the lungs, where they contributed to the formation of metastatic lesions.

In our study, men with detectable levels of circulating fibroblast-like cells had cell numbers ranging from 2 to 12 per 7.5 ml of blood (Fig. 1D). These cell numbers are low, possibly due to the limited sensitivity of the CellSearch® method [21]. Future work should focus on evaluating the presence of circulating fibroblast-like cells using a technique that is more sensitive, such as the recently developed Herringbone–Chip method [22], and more direct (i.e., directly capture circulating fibroblast-like cells rather than $EpCAM^+$ cells and cells that have adhered to $EpCAM^+$ cells). Future studies should also involve larger numbers of study participants.

CONCLUSIONS

Overall, the results of our study suggest that the presence of fibroblast-like cells in the blood may distinguish men who have metastatic prostate cancer from men who do not. Thus, these cells may have potential as a prognostic marker and/or therapeutic target in men with prostate cancer. Further work needs to be done to evaluate the clinical utility of circulating fibroblast-like cells, including their ability to predict a poor outcome, and to better understand their biology, that is, where they came from, where they end up, and how they contribute to metastatic progression.

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