Characterization of *ERG*, *AR* and *PTEN* Gene Status in Circulating Tumor Cells from Patients with Castration-Resistant Prostate Cancer

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Abstract

Hormone-driven expression of the *ERG* oncogene after fusion with *TMPRSS2* occurs in 30% to 70% of therapy-naive prostate cancers. Its relevance in castration-resistant prostate cancer (CRPC) remains controversial as *ERG* is not expressed in some *TMPRSS2-ERG* androgen-independent xenograft models. However, unlike these models, CRPC patients have an increasing prostate-specific antigen, indicating active androgen receptor signaling. Here, we collected blood every month from 89 patients (54 chemotherapy-naive patients and 35 docetaxel-treated patients) treated in phase I/phase II clinical trials of an orally available, highly specific CYP17 inhibitor, abiraterone acetate, that ablates the synthesis of androgens and estrogens that drive *TMPRSS2-ERG* fusions. We isolated circulating tumor cells (CTC) by anti–epithelial cell adhesion molecule immunomagnetic selection followed by cytokeratin and CD45 immunofluorescence and 4′,6-diamidino-2-phenylindole staining. We used multicolor fluorescence *in situ* hybridization to show that CRPC CTCs, metastases, and prostate tissue invariably had the same *ERG* gene status as therapy-naive tumors (n = 31). We then used quantitative reverse transcription–PCR to show that *ERG* expression was maintained in CRPC. We also observed homogeneity in *ERG* gene rearrangement status in CTCs (n = 48) in contrast to significant heterogeneity of *AR* copy number gain and *PTEN* loss, suggesting that rearrangement of *ERG* may be an earlier event in prostate carcinogenesis. We finally report a significant association between *ERG* rearrangements in therapy-naive tumors, CRPCs, and CTCs and magnitude of prostate-specific antigen decline (P = 0.007) in CRPC patients treated with abiraterone acetate. These data confirm that CTCs are malignant in origin and indicate that hormone-regulated expression of *ERG* persists in CRPC. [Cancer Res 2009;69(7):2912–8]

Introduction

Hormone-driven expression of the *ERG* oncogene after fusion with *TMPRSS2* occurs in 30% to 70% of therapy-naive prostate cancers (1). Its relevance in castration-resistant prostate cancer (CRPC) remains controversial, as *ERG* is not expressed in some *TMPRSS2-ERG* androgen-independent xenograft models (2). However, unlike these models, CRPC patients have an increasing prostate-specific antigen (PSA) indicating active androgen receptor (AR) signaling. Recent studies indicate that the oral and highly specific CYP17 inhibitor, abiraterone acetate, ablates the synthesis of androgens and estrogens that drive *TMPRSS2-ERG* fusions (1, 3), inducing disease regression in >50% of CRPC patients (4). We hypothesized that hormone-dependent overexpression of ERG persisted in CRPC and that *TMPRSS2-ERG* tumors represented a subgroup of prostate cancers that remained sensitive to CYP17 blockade. We also hypothesized that two potential mechanisms of resistance to CYP17 were gain of *AR* and loss of *PTEN* that could result in constitutive phosphorylation of the AR, leading to ligandless activation (5–7).

We have used anti–epithelial cell adhesion molecule (EpCAM) antibody-based immunomagnetic selection to isolate CRPC circulating tumor cells (CTC; ref. 8) on which to perform multicolor fluorescence *in situ* hybridization (FISH) investigating the *ERG, AR*, and *PTEN* gene loci. As considerable differences exist in how a CTC is defined, with several groups reporting these events differently (9–11), we describe all anti-EpCAM and cytokeratin (CK)–positive categories.

Materials and Methods

Patients

Blood (7.5 mL) was collected into a CellSave tube (Immunicon) from patients with biochemically or histologically confirmed prostate cancer and an increasing PSA despite castrate levels of testosterone (<20 ng/dL) before starting and at monthly intervals until progression on clinical trials of abiraterone acetate. CTCs were enumerated by the CellSearch System (Veridex LLC; ref. 8). Archival tumor acquired before administration of any hormone treatment and blood for CTC analysis were prospectively collected, and patients with untreated primary tumors or accessible metastatic disease were given the option to undergo a tumor biopsy for research purposes. Samples were anonymized by the study trial coordinators, and operators were blinded to clinical outcome. This study was approved by the Royal Marsden NHS Hospital Ethics Review Committees, and informed consent was obtained for these studies.

FISH. Four-color FISH using an *ERG* break-apart assay (12) and probes covering the *AR* and the minimal region of deletion at the *PTEN* gene locus

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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(Supplementary Fig. S1; ref. 13) was performed on CTCs fixed in cartridges with 3:1 methanol/acetic acid. Briefly, CTCs were hybridized overnight at 42°C with the hybridization mix containing rhodamin-labeled *PTEN* probes, Dy647-labeled 3′-*ERG*, F415-labeled 5′-*ERG*, and FITC-labeled *AR*. Cartridges were washed twice with 24% formamide/0.1× SSC at 42°C and then filled with PBS. Cells were then individually captured with a 40× objective in five Z-stack images over 8 μm for each filter (14). Flattened images were scored for whether they had loss of half or all copies of *PTEN* (heterozygous or homozygous loss) and increased *AR* copy number and, for *ERG* probes, whether they were (*a*) class "Normal," with unrearranged *ERG* loci characterized by twinned Dy415 (in red; 3′-*ERG*) and Dy647 (in green; 5′-*ERG*) FISH signals; (*b*) class "Esplit," with a rearranged *ERG* locus manifested as separation of the red and green signals; or (*c*) class "Edel" (for *ERG* 5′-deletion), an *ERG* rearrangement with loss of green signal corresponding to 5′-*ERG* sequences. Within classes Edel and Esplit, the pattern could be further divided into cancers that contained one copy of the red 3′-*ERG* signal ("1 Edel" and "1 Esplit") and those that exhibit more than one copy of the 3′-*ERG* FISH signal ("2+ Edel" and "2+ Esplit") (12). Class Normal tumors were classified as 3+ Normal if the presence of three or more copies of wild-type (WT) *ERG* were observed (Supplementary Table S1). For *PTEN* loss and *ERG* gene status, patients were classified into the potentially more aggressive class, i.e., homozygous versus heterozygous loss and 2+ Edel or 2+ Esplit versus Edel or Esplit.

FISH using an *ERG* break-apart assay was also performed, as described previously (12), on tissue slices adjacent to H&E sections confirmed to contain cancer. All the tissues were then scanned using an Ariol SL-50 scanner (Applied Imaging), and all areas of tumor were scored for *ERG* gene status. Tumors were classified by *ERG* gene class, as used for CTCs (Supplementary Table S1).

Expression studies. Total RNA was extracted from formalin-fixed, paraffin-embedded samples using the RNeasy kit (Qiagen) and frozen samples using Trizol (Invitrogen) following manufacturer's instruction and quantified by UV spectroscopy. cDNA was generated by reverse transcription–PCR (RT-PCR) using first-strand cDNA synthesis system. Relative mRNA levels were determined by quantitative-PCR (qPCR; 7900HT Fast Real-Time PCR System, Applied Biosystems). ERG mRNA levels were measured using primers spanning the exon 9/10 boundary (forward, 5′-GAACGAGCGCAGAGTTATCGT-3′; reverse, 5′-TGCCGCACATGGTCTGTACT-3′) expressed relative to rpl32 mRNA levels (forward, 5′-CCCTTGTGAAGCCCAAGA-3′;reverse, 5′-GACTGGTGCCGGATGAACTT-3′) and normalized to the ERG/rpl32 ratio of VCaP cells cultured in 10% fetal bovine serum (FBS)–containing media. Dextran-activated charcoal-stripped FBS (CSS) or CSS plus 10^{-9} M R1881 for 48 h. All qPCR assays were performed in quadruplicate.

Results

Detecting CTCs in CRPC. Blood was collected from 89 CRPC patients being treated in phase I/II clinical trials of abiraterone acetate; 54 patients were chemotherapy-naive and 35 had received docetaxel. Overall, most patients had metastatic disease with only four chemotherapy-naive patients having an increasing PSA with no radiological evidence of disease on computed tomography scans or bone scan (Table 1). All patients had CK+ and CK+ CD45− events with relatively few being classified with an intact nucleus and cytoplasm (Supplementary Table S2). Of these intact CTCs, those that are undergoing apoptosis did not provide a FISH signal (Supplementary Table S3). A minimum of FISH results on four CTC was required to ensure that the result was technically reproducible, as the evaluation of FISH scores in 1,000 WBC controls revealed a 3.6% and 4.3% false-positive rate for the presence of *ERG* gene rearrangements and heterozygous *PTEN* loss, respectively (36/1000 WBC had splitting or loss of *ERG* locus probe and 42/1000 WBC had loss on one *PTEN* locus probe in a nucleus; Supplementary Table S4). Four or more CTC events were identified at any time point on the study in 51 patients, all of whom had radiologically detectable metastatic disease. The number of CTCs declined with treatment with abiraterone acetate (Supplementary Fig. S2; Supplementary Table S5) and was concordant in this cohort of patients with changes in PSA (correlation coefficient, $r = 0.730$; $P < 0.0001$, Spearman rank test; Supplementary Fig. S3). We confirmed, in this cohort of patients, previous observations that the number of CTCs is associated with survival and that a decline in the counts after treatment was associated with an improvement in outcome (Supplementary Figs. S4 and S5; refs. 15–17).

Table 1. Patient characteristics divided by prior chemotherapy treatment

	Chemotherapy naive	Docetaxel treated
Total no. patients	54	35
Bone metastasis	41 (75%)	33 (94%)
Soft tissue metastasis	31 (57%)	21 (60%)
No radiologically detectable metastases	4 (7%)	0
Median pretreatment PSA (ng/mL)	88 (range, 8.8–964)	501 (range, 26.4–10,325)
CTC of ≥4 at any time point on study	22 (40%)	29 (82%)
FISH results on CTC	20	29
ERG rearranged	8 (40%)	15 (51%)
Class Edel	5 (62%)	10 (66%)
Class Esplit	3 (38%)	5 (34%)
PTEN loss	6 (30%)	7 (24%)
Homozygous	4	5
Heterozygous	2	2
AR copy number gain		
4/5 copies of *AR* (maximum)	2 of 11 (18%)	8 of 22 (36%)
>5 copies of *AR* (maximum)	7 of 11 (63%)	8 of 22 (36%)

NOTE: *ERG* and *PTEN* CTC gene status was available for 49 of 51 patients (96%) with four or more CTCs at any time point on study. *AR* copy number was evaluated in 33 of 51 patients (64%) with four or more CTCs at any time point on study (maximum copy number observed is reported).

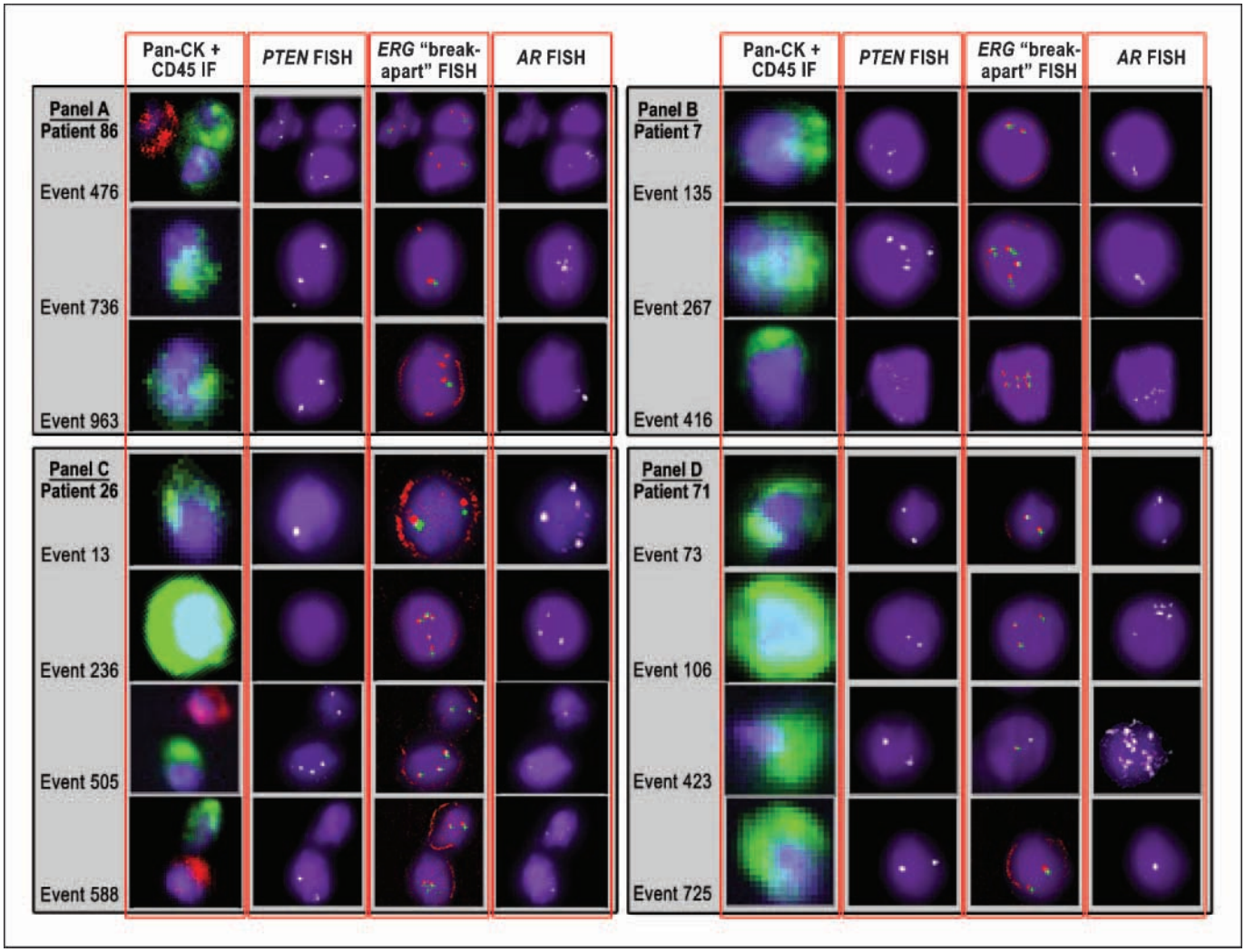

Figure 1. Molecular characterization of CTCs. CK (*green*) and CD45 (*red*) immunofluorescence and *PTEN, ERG* break-apart, and *AR* FISH. Examples of events from four patients that were CK positive and had an intracellular 4′,6-diamidino-2-phenylindole–stained nucleus are included. There are three examples of control leukocytes that stain positive for CD45 (*red*) and negative for CK (*no green*; *A,* event 46; *C,* events 505 and 588). *A,* class Edel with *ERG* gene copy number heterogeneity. Patient 86 had loss of the 5′-*ERG* signal suggesting fusion of *TMPRSS2* with *ERG* secondary to deletion. All CTC analyzed were class Edel. All events had one copy of WT *ERG*, but event 476 has two copies of rearranged *ERG*, event 736 has one copy, and event 963 has three copies. Event 476 has three copies of *AR* and three of *PTEN*, event 736 has five copies of *AR* and two of *PTEN*, and event 936 has two copies of *AR* and two of *PTEN*. *B, ERG* WT ploidy. Patient 7 did not have rearrangement of *ERG* in any CTC. Event 135 has two copies of WT *ERG* and three copies of *PTEN*, event 267 has four copies of WT *ERG* and five of *PTEN*, and event 416 has six copies of WT *ERG* and eight copies of PTEN. The number of copies of AR does not exceed the number of copies of *PTEN* or WT *ERG* in any event shown, but seven CTC with more than five copies of AR but two of three copies of *PTEN* and WT *ERG* were observed (not shown). *C,* heterogeneous *PTEN* loss. Patient 26 is a case of homozygous PTEN loss as shown in events 236 and 588; the CTC in event 588 is adjacent to a control leukocyte with two copies of *PTEN*. There is loss of one copy of *PTEN* in event 13 and normal *PTEN* complement in event 505. *ERG* is not rearranged in any CTC; there are two copies of WT *ERG* in events 13 and 588, five copies in event 236, and three copies in event 505. There are seven copies of *AR* in event 13, three copies in event 236, one copy in event 505, and two copies in event 588; no other CTC had more than five *AR* copies. *D,* heterogeneous *AR* gain. Patient 71 has two or three copies of WT *ERG* and *PTEN* in all CTCs. Event 725 has one copy of *AR*, event 73 has two copies of *AR*, event 106 has six copies of *AR*, and event 423 has >10 copies of *AR*.

FISH on CTCs reveals homogenous *ERG* rearrangements but heterogenous gain of *AR* and loss of *PTEN*. FISH was successfully performed on CTCs from 49 of 51 patients (96%) who had four or more CTCs [20 of 54 (37%) chemotherapy-naïve patients; 29 of 35 (82%) docetaxel-treated patients]. *ERG* was rearranged in CTCs from 23 of 49 patients (47%); 15 of 23 (65%) from class Edel and 8 of 23 (34%) from class Esplit (Table 1 and Supplementary Table S6). Previous studies investigating *TMPRSS2-ERG* in CRPC used materials collected at rapid autopsy from a smaller number of patients and failed to detect any class Esplit tumors (18). In all our cases, no patient had four or more CTCs belonging to different *ERG* gene classes; no cases of CTCs with unrearranged *ERG* were observed in patients with an *ERG* rearrangement in biopsy tissue or CTCs. Also, in patients with an *ERG* rearrangement, no cases had four or more CTCs classified both class Edel and class Esplit. More than one copy of rearranged *ERG* was observed in 10 patients (2+ Edel or 2+ Esplit, 20% of all patients; Figs. 1*A* and 2*A*), and 13 patients (26%) had CTCs with two or more copies of WT *ERG* (3+ Normal; Fig. 1*B*, 2*B*; *ERG* gene classes are explained in Supplementary Table S1). In all patients with more than two copies of the *ERG* gene locus, copy number was gained heterogeneously with single patients having CTCs with a range of different copy numbers (Fig. 2*B*). More than two copies of the *ERG* gene locus were associated with more than two copies

of *PTEN* in patients with no *PTEN* loss. In contrast, FISH studies revealed significant heterogeneity of *PTEN* loss and *AR* copy number gain. Thirteen patients (26%) had evidence of loss of *PTEN* in their CTCs (Table 1). Six of 13 patients had homogenous homozygous loss of *PTEN* in all their CTCs. Seven patients had heterogeneous *PTEN* loss: two patients each had four or more CTCs with homozygous and heterozygous loss and four or more cells with a full complement of *PTEN* (Fig. 1*C*), one patient had four or more CTCs harboring both heterozygous and homozygous *PTEN* loss, and four patients had four or more CTCs with both heterozygous loss and normal *PTEN* (Fig. 2*C*). All cases with heterozygous loss (n = 7) showed heterogeneity of *PTEN* loss but only three of nine with homozygous loss showed heterogeneity. *AR* gene copy number was available on CTCs for 33 patients: copy number gain was invariably heterogeneous (Fig. 1*D*). No patient had only one copy of *AR* in all their CTCs, whereas WBCs all revealed one *AR* copy. Twenty-eight patients (85%) had CTCs harboring a maximum of three copies of *AR*, 10 patients (30%) had CTCs with a maximum of four or five copies, and 15 patients (45%) had CTCs with more than five copies of *AR* (Supplementary Table S7). There was no change in *ERG* and *PTEN* status in CTCs collected from patients before starting and after progressing on abiraterone acetate (n = 24). Similarly, there was no shift in *AR* copy number with treatment with abiraterone acetate (n = 18; Supplementary Table S7).

***ERG* gene status does not change with the development of castration resistance.** We then proceeded to compare the *ERG* gene status of CTCs with that of archival diagnostic tumor tissue acquired before the institution of first-line hormonal treatment (n = 27) and of CRPC biopsies (n = 11) from the same patient. We found that CTC *ERG* gene status matched the *ERG* gene status of tumor tissues in all cases (Table 2). These data also showed that all patients with a rearrangement of *ERG* in the therapy-naive tissue had *ERG*-rearranged cancer in the CRPC tissue; similarly, no CRPC tissue with *ERG* rearrangements was detected in patients who did not have a rearrangement at baseline (n = 31). One patient (patient 74) had class Normal and class Edel in his baseline therapy-naive samples; all his CTCs were class Edel. One patient (patient 70) had 70% class Edel and 30% class Esplit in his therapy-naive samples; his CTCs were class Edel. Similarly, a second patient (patient 2) had 65% class Esplit and 35% class Edel in his therapy-naive samples; his CTC were class Esplit.

***ERG* remains expressed in CRPC.** To confirm that ERG expression was maintained in CRPC samples with a *TMPRSS2-ERG* fusion detectable by FISH (break-apart assay), quantitative RT-PCR was performed for ERG exon 11 in CRPC tumor samples, and this was matched with therapy-naive samples. Expression of *ERG* exon 11 was significantly higher in all *ERG*-rearranged CRPC samples (n = 9) compared with CRPC samples with no *ERG* rearrangement (n = 6; P = 0.0004, Mann-Whitney test). Post–abiraterone acetate treatment biopsies were performed in three patients, but tumor tissue was identified in only one patient. A decrease in expression of ERG exon 11 was observed in this patient while responding to abiraterone acetate. Similarly, androgen-responsive expression of ERG exon 11 increased 3.5-fold in VCaP cell lines treated with R1881 (Fig. 3; Supplementary Table S8).

Clinical implications of assessing *ERG* gene status. As there was no change in *ERG* gene status with castration resistance or with abiraterone acetate treatment, we combined *ERG* gene FISH data from CTCs (n = 49), CRPC tumor tissue (n = 17), and archival, therapy-naive tissue (n = 53) to obtain an *ERG* gene class for 77 patients (we have been unable to acquire neither tumor tissue nor CTC for the remaining 12 patients). Thirty-two patients (41%) had an *ERG* rearrangement, and 45 did not. Nineteen patients were on a stable dose of steroids at the start of the study and continued the same steroid dose in combination with abiraterone acetate, and 58 patients were treated with single-agent abiraterone acetate. All patients were participating in phase I/phase II clinical trials with a primary efficacy end point of PSA decline as recommended by PSA working group I criteria (19). The presence of an *ERG* rearrangement was associated with the magnitude of maximal PSA decline on abiraterone acetate (P = 0.007, Mann-Whitney test for trend), with 12 of 15 patients (80%) who had a ≥90% PSA decline, having an *ERG* rearrangement compared with 20 of 62 patients (32%) who did not have a ≥90% PSA decline being *ERG* rearranged (P = 0.001, Fisher exact test). The association between an *ERG* rearrangement and declines in PSA of a smaller magnitude than 90% approached

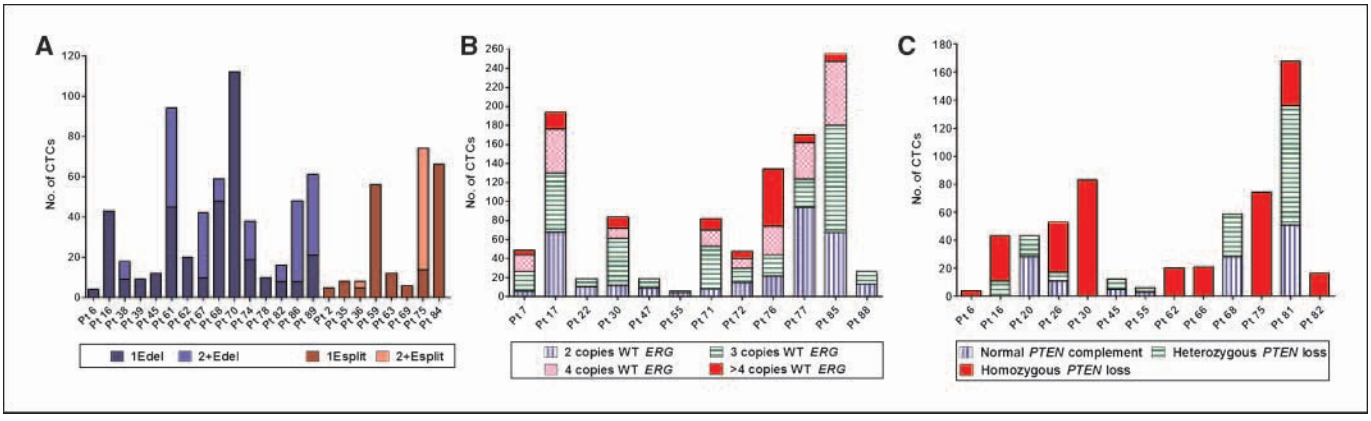

Figure 2. Homogeneity of *ERG* gene rearrangement and heterogeneity of *PTEN* loss and *AR* copy number gain. CTC with FISH abnormalities in three or fewer CTCs are not represented *A, ERG* gene copy number of all 23 patients with rearrangement. Fifteen patients are of class Edel, and eight are of class Esplit; eight cases had CTC harboring both 1 Edel and 2 Edel, and two cases had CTC harboring both 1 Esplit and 2+ Esplit. No patient had four or more CTC belonging to more than one *ERG* gene class. *B,* WT *ERG* gene ploidy (class 3+ Normal). Twelve patients had CTC harboring three or more copies of WT *ERG*. *C,* heterogeneity of *PTEN* loss. Thirteen patients had CTC with evidence of loss of PTEN. Six patients had homogenous homozygous loss of both PTEN loci. The other seven patients had CTC with heterogeneous loss of *PTEN*.

significance (association with ≥50%, P = 0.07, Fisher exact test; Table 3).

Discussion

The 100% concordance in *ERG* gene status of CRPC (tumor biopsies and CTCs) and therapy-naive tissues acquired several years and treatments previously suggests that, despite multiple foci of cancer with different *ERG* gene status existing in a single prostate (20, 21), the cancer that is most commonly detected on *trans*-rectal biopsy of the prostate will result in blood-borne metastases after progression on castration. These data suggest that *TMPRSS2-ERG* tumors may represent a subgroup of prostate cancers that are enriched for tumors that remain exquisitely sensitive to CYP17 blockade. However, 12 of 32 *TMPRSS2-ERG* tumors (37%) did not have a ≥50% PSA decline, suggesting that they had primary resistance to abiraterone acetate. Moreover, hormone-dependent fusions of *ETV1, ETV4*, and *ETV5*, although less common, could account for abiraterone-sensitive *ERG*-unrearranged cancers (1, 22, 23). As PSA is an androgen-regulated gene, the association observed between PSA declines, the primary end point of these studies, and *ERG* rearrangements may not translate into an improvement in overall survival: this will require further evaluation in larger, future studies.

Table 2. *ERG* gene status in matched therapy-naive and castration-resistant tumor tissue

Patient no.	Therapy-naive cancer (no. TRBP cores, % cancer involvement)	Castration-resistant cancer: Tumor biopsies (site, no. cores, % cancer involvement)	CTCs (no. of CTCs scored)
1	1 core, 60%		6
2	3 cores, 60%		11
6	10 cores, 100%*	1 liver biopsy, 60%*	4
7	5 cores, 60%*	2 TRBP cores, 40%*	49
14	1 core, 60%	1 TRBP core, 40%	
15	5 cores, 80%	PUT (multiple chips)	
16	1 core, 60%		38
17	8 cores, 80%		184
18	1 core, 60%	1 core, 60%*	
26	4 cores, 100%		53
28	TURP (multiple chips)	TURP (multiple chips)*	253
30	5 cores, 60%		83
35		1 TRBP core, 80%*	8
36	3 cores, 80%*	2 TRBP cores, 40%*	8
38	3 cores, 70%		18
39	1 core, 40%		9
43	1 core, 60%		205
44	TURP (multiple chips)*	4 liver biopsies, 90%*	
47	2 cores, 100%		19
55	3 cores, 30%		6
59	1 core, 40%		56
60	3 cores, 70%		81
61	1 core, 80%		94
63		1 TRBP, 40%*	12
64	1 core, 60%		23
66	1 core, 100%	1 TRBP core, 30%*	21
67		1 lymph node biopsy, 70%	54
69	TURP (multiple chips)*	1 TRBP core, 30%*	6
70	5 cores, 80%		112
71	1 core, 60%		82
72	1 core, 100%		58
73	2 cores, 80%		20
74	1 core, 60%*	2 TRBP, 70%*	38
77	1 core, 100%		170
87		1 TRBP core, 30%*	6

Key: , class Normal; , class Edel; , class Esplit; , class 2+ Edel; class 2+ Esplit; TRBP, *trans*-rectal biopsy of the prostate; TURP, *trans*-urethral resection of the prostate; PUT, paraureteric tumor.
*Quantitative RT-PCR performed on RNA (Fig. 3).

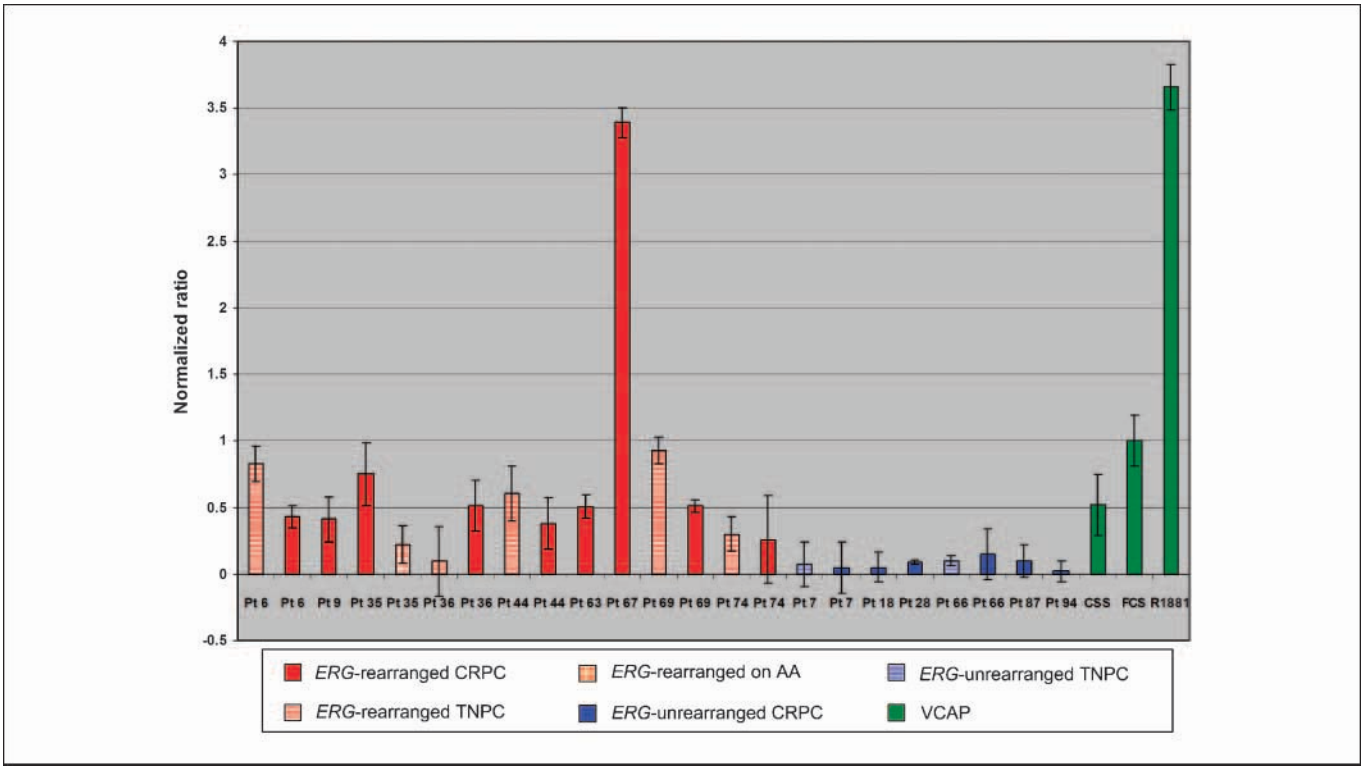

Figure 3. *ERG* remains expressed in CRPC. The bar chart represents expression of tumor samples obtained from patients treated on clinical studies of abiraterone acetate, normalized to the expression of *ERG* by VCAP cells in normal serum. Columns in red on the left side of the chart represent patients with an *ERG* rearrangement confirmed by FISH and a *TMPRSS2-ERG* fusion transcript confirmed by RT-PCR. Columns in blue in the middle of the chart represent patients with no *ERG* rearrangement. Striped columns signify a therapy-naive sample matched to a castration-resistant sample. One patient had castration-resistant samples collected immediately before starting abiraterone acetate and a second set of samples acquired while receiving treatment with abiraterone acetate (*column with diamond pattern*). The columns in green on the right side of the chart represent expression of *ERG* by VCaP cells in charcoal-stripped serum, FBS, and added synthetic androgens (R1881). *Bars,* SE. The maximal percentage of PSA decline, and time-on-treatment on abiraterone acetate for these patients is reported in Supplementary Table S8. *TNPC,* therapy-naive prostate cancer; *AA,* abiraterone acetate; *CSS,* charcoal-stripped serum; *R1881,* synthetic androgen added to serum.

Novel antiandrogens that target the AR and small molecule inhibitors of the phosphatidylinositol-3-kinase (PI3K)/PTEN are being evaluated in clinical trials as a strategy for reversing resistance to hormone therapies (6). However, of the patients with evidence of *PTEN* loss, 7 of 13 had ≥50% declines in PSA and one (who had 100% homozygous *PTEN* loss and a class 2+ Edel *ERG* rearrangement) had a ≥90% PSA decline. Similarly, tumor responses were observed in patients with *AR* copy number gain. Moreover, loss of *PTEN* was not observed at the development of resistance to abiraterone acetate. As this study reports multiple subclasses for every gene locus, for example, homozygous *PTEN* loss in 100%, 80% to 100%, 60% to 80%, 40% to 60%, and<40% of CTCs, and *ERG* gene rearrangements by deletion or rearrangement and with or without duplication, large numbers of patients need to be evaluated to investigate the effect of specific genetic aberrations on clinical outcome. This will be pursued in an ongoing randomized double-blind phase III trial of abiraterone acetate and prednisone versus prednisone and placebo. The significant genetic heterogeneity observed at the *PTEN* and *AR* loci contrasts to *ERG* homogeneity and, together with the absence of a change in *ERG* gene status with the development of castration resistance, could suggest that rearrangement of *ERG* is an earlier event in prostate carcinogenesis than gain of *AR* and, in some cases, loss of *PTEN*. Moreover, CTC genetic heterogeneity for *AR* amplification and *PTEN* loss may have important implications for the development of therapeutics that target either the AR or PI3K/AKT pathways. Finally, these data strongly suggest that circulating, non-apoptotic nucleated, EPCAM+ CK+ CD45- cells isolated from CRPC patients are malignant in origin and confirm that hormone-regulated expression of *ERG* persists in CRPC.

Disclosure of Potential Conflicts of Interest

All patients participating in clinical trials were sponsored by Cougar Biotechnology. Processing of blood samples for CTC evaluation was conducted in a research collaboration with Immunicon Corporation. However, neither Cougar Biotechnology nor Immunicon Corporation had any input to the study design, analysis of samples, or interpretation of the data. J.F. Swennenhuis, R. Levink, F. Coumans, R. Sipkema, and L.W.M.M. Terstappen were employees of Immunicon Corporation, which has been purchased by Veridex LLC. Veridex LLC owns the rights to CTC evaluation using the technology described in this manuscript. L.W.M.M. Terstappen is a consultant for Veridex LLC. A. Molina is a full-time employee of Cougar Biotechnology, which holds the rights of development of abiraterone acetate. J.S. De Bono has served as unpaid consultant for Cougar Biotechnology. Abiraterone acetate was discovered at The Institute of Cancer Research, which therefore has a commercial interest in the development of this agent. G. Attard, D. Olmos, A.H.M. Reid, R. A'Hern, J. Moreira, R. Riisnaes, E. Thompson, C.P. Carden, C. Parker, D. Dearnaley, S.B. Kaye, C.S. Cooper, J.S. De Bono are employees of The Institute of Cancer Research.

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Table 3. *ERG* rearrangements are associated with magnitude of PSA decline on abiraterone acetate

	ERG rearranged	*ERG* normal	Total
I			
No	20	42	62
≥90% decline	12	3	15
Total	32	45	77
			$P = 0.001$
II			
No	12	27	39
≥50% decline	20	18	38
Total	32	45	77
			$P = 0.07$
III			
No	12	27	62
50–89%	8	15	38
≥90% decline	12	3	15
Total	32	45	77
			$P = 0.007$

NOTE: The number of patients with a PSA decline of ≥90% (I), ≥50% (II), and ≥50–89% and ≥90% (III) are divided by *ERG* gene status.

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G. Attard and J.S. De Bono designed the study, interpreted the data, and wrote the first draft of the manuscript. J.F. Swennenhuis, R. Levink, F. Coumans, R. Sipkema, and L.W.M.M. Terstappen developed the technology for CTC identification and FISH on CTC, performed FISH on all the CTCs, and assisted G. Attard with the analysis of the samples. E. Vickers and M.E. Cox assisted G. Attard with the experiments for analysis of *ERG* expression and, with G. Attard and J.S. De Bono, interpreted the results. G. Attard, J.S. De Bono, D. Olmos, A.H.M. Reid, N. Babu Oommen, C.P. Carden, C. Parker, D. Dearnaley, and S.B. Kaye contributed patients to this study. G. Hawche and E. Thompson assisted with collection of the samples. J. Moreira and C.S. Cooper assisted G. Attard with FISH on tissue samples. C. Jameson scored all samples for areas of cancer and contributed expert pathologic advice. R. Riisnaes sectioned all the tumor samples. A. Molina was involved in the administration of the abiraterone acetate studies on behalf of Cougar Biotechnology. R. A'Hern and D. Olmos gave expert statistical advice on interpreting the results. All authors contributed to the final version of the manuscript.

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