PLZF, a Tumor Suppressor Genetically Lost in Metastatic Castration-Resistant Prostate Cancer, Is a Mediator of Resistance to Androgen Deprivation Therapy

Chen-Lin Hsieh[1], Ginevra Botta[1,3], Shuai Gao[4], Tiantian Li[1], Eliezer M. Van Allen[1,2], Daniel J. Treacy[1], Changmeng Cai[4], Housheng Hansen He[5,6], Christopher J. Sweeney[1], Myles Brown[1,2], Steven P. Balk[4], Peter S. Nelson[7], Levi A. Garraway[1,3], and Philip W. Kantoff[1]

Abstract

Whole-exome sequencing of metastatic castration-resistant prostate cancer (mCRPC) reveals that 5% to 7% of tumors harbor promyelocytic leukemia zinc finger (PLZF) protein homozygous deletions. PLZF is a canonical androgen-regulated putative tumor suppressor gene whose expression is inhibited by androgen deprivation therapy (ADT). Here, we demonstrate that knockdown of PLZF expression promotes a CRPC and enzalutamide-resistant phenotype in prostate cancer cells. Reintroduction of PLZF expression is sufficient to reverse androgen-independent growth mediated by PLZF depletion. PLZF loss enhances CRPC tumor growth in a xenograft model. Bioinformatic analysis of the PLZF cistrome shows that PLZF negatively regulates multiple pathways, including the MAPK pathway. Accordingly, our data support an oncogenic program activated by ADT. This acquired mechanism together with the finding of genetic loss in CRPC implicates PLZF inactivation as a mechanism promoting ADT resistance and the CRPC phenotype. *Cancer Res; 75(10); 1944–8. ©2015 AACR.*

Introduction

A long-standing challenge in the management of prostate cancer is the development of resistance to androgen deprivation therapy (ADT), a standard treatment to disrupt the androgen receptor (AR) signaling pathway, because AR has a profound effect on prostate carcinogenesis through the regulation of transcriptional networks, genomic stability, and gene fusions (1). Although ADT is initially effective and presumably extends the survival of most prostate cancer patients, prostate cancer inevitably becomes resistant to ADT and castration-resistant prostate cancer (CRPC) emerges (2). Newer agents targeting the androgen signaling axis (AR-targeted therapies), such as abiraterone and enzalutamide, have yielded improved outcomes for patients with CRPC. Unfortunately, not all patients with CRPC respond to these AR-targeted therapies, and moreover, these agents are not curative in this setting (3). The main subset of mechanisms of resistance to these antagonists involves the AR signaling pathway, including AR gene overexpression, gain-of-function mutations, constitutively active AR splice variants, dysregulation of its coregulators, and *de novo* androgen synthesis (4). Additional categories of resistance mechanisms consist of de-repression of progrowth pathways in response to ADT (5) or transformation to a distinct, androgen, and AR-indifferent cell state (4).

The recent surge of genomic and transcriptomic information may permit molecular classification of CRPC and future clinical development of precision medicine based on predictive biomarkers (5). Intriguingly, whole-exome sequencing of metastatic CRPC (mCRPC) revealed that 5% to 7% of tumors harbor promyelocytic leukemia zinc finger (PLZF) focal homozygous deletions. PLZF, also known as BTB-containing protein 16 (*ZBTB16*), was originally identified as a gene fused to RARα in acute promyelocytic leukemia patients (6). PLZF has been shown to play an important role in the regulation of major developmental and biologic processes and carcinogenesis as a tumor suppressor gene, since it regulates the cell cycle and apoptosis in various cell types (7). Overexpression of PLZF was shown to inhibit cellular proliferation in AR-positive LNCaP and AR-negative DU-145 prostate cancer cell lines (8, 9). Herein, our data show that PLZF emerged as the top gene from an AR cistrome analysis, credentialing PLZF as an androgen-regulated putative tumor suppressor gene in prostate cancer. Accordingly, we report a

Department of [1]Medical Oncology, and [2]Center for Functional Cancer Epigenetics, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. [3]Broad Institute, Cambridge, Massachusetts. [4]Hematology-Oncology Division, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts. [5]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada. [6]Princess Margaret Cancer Centre, University Health Network, Toronto, Ontario, Canada. [7]Division of Clinical Research, Fred Hutchinson Cancer Research Center and Department of Medicine, University of Washington School of Medicine, Seattle, Washington.

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

C.-L. Hsieh and G. Botta contributed equally to this article.

Corresponding Authors: Philip W. Kantoff, Dana-Farber Cancer Institute, Suite D1230, 450 Brookline Avenue, Boston, MA 02215. Phone: 617-632-1914; Fax: 1-617-632-2165; E-mail: philip_kantoff@dfci.harvard.edu; or Levi A. Garraway, levi_garraway@dfci.harvard.edu

doi: 10.1158/0008-5472.CAN-14-3602

AACR

resistance mechanism to ADT mediated by PLZF, which appears to result from the activation of progrowth pathways in response to ADT. Furthermore, the findings of PLZF genetic loss in mCRPC tumors support that PLZF may be an important mediator in a subset of CRPC tumors.

Materials and Methods

Cell culture, lentiviral infection, and xenografts

LNCaP/22Rv1 and VCaP cells were cultured in RPMI1640 and DMEM medium with 10% FBS. 22Rv1 xenografts were established in the flanks of male nude mice by injecting approximately 2 million stable 22Rv1 cells with shCtrl or shPLZF knockdown in 50% Matrigel 3 days after castration. Tumors were measured 3 times every week and harvested after 3 weeks. All animal experiments were approved by the Beth Israel Deaconess Institutional Animal Care and Use Committee and were performed in accordance with institutional and national guidelines

Cell proliferation (crystal violet staining/WST1)

Cell growth was examined using the crystal violet (CV) staining and WST1 assays (Roche) following the manufacturer's protocol. CV was dissolved in 10% acetic acid and cell proliferation calculated relative to the negative control cells, by measuring the absorbance at 595 nm.

qRT-PCR, immunoblotting, and immunohistochemistry

RNAs were extracted using TRIzol according to the manufacturer's protocol. Primers are listed in Supplementary Text. qPCR data are represented as mean ± STD for more than 3 replicates. Blots were incubated with anti-PLZF (MAB2944; R&D Systems), anti-actin (A5316; Sigma), total p44/42 MAK (Erk1/2; 4695; Cell Signaling), or phospho-p44/42 MAK (Erk1/2; 4370; Cell Signaling). Paraffin sections underwent antigen retrieval and were subjected to the staining protocol using Dako EnVision+System-HRP 3,3′-diaminobenzidine (DAB). Anti-PLZF (MAB2944; R & D

Systems), anti-Ki67 (Dako), or nonspecific IgG was then added overnight at 4°C. Sections compared in each figure were stained at the same time and photographed under identical conditions.

Chromatin immunoprecipitation assay and ChIP-Seq data analysis

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (10). The PLZF antibody (MAB2944; R&D Systems) or nonspecific IgG was used for ChIP. ChIP-Seq raw data were mapped by Bowtie 2 with default parameters. The identification of ChIP-seq peaks (bound regions and summit) was performed using MACS (PMID: 18798982). Regions of enrichment comparing to input control exceeding a given threshold ($P < 1e-5$) were called as peaks. The primers for qPCR are provided in the Supplementary Text.

Gene expression experiments and analysis

LNCaP cells were transfected with either control shRNA (shCtrl) or shRNAs targeting PLZF (shPLZF). Forty-eight hours after shRNA transfection, total RNA was isolated and hybridized to Affymetrix human U133 plus 2.0 expression array (Affymetrix). Raw data are preprocessed using RMA (PMID: 12538238) and the cutoff of 1.5-fold change, and P value of <0.05 is applied for differential expressed gene analysis.

Results and Discussion

The hope of precision medicine is to tailor treatment based on each patient's genomic and transcriptomic characteristics. This approach has proven to be challenging for the management of prostate cancer because of the paucity of actionable mutations found thus far. The recent finding (11) that 7% (4/61) of mCRPC tumors harbored PLZF homozygous deletions captured our interest (Fig. 1A). Indeed, homozygous deletion of PLZF was further seen in two independent cohorts: 6% (4/63) and 5% (8/152) from the University of Washington and the Stand Up to Cancer/
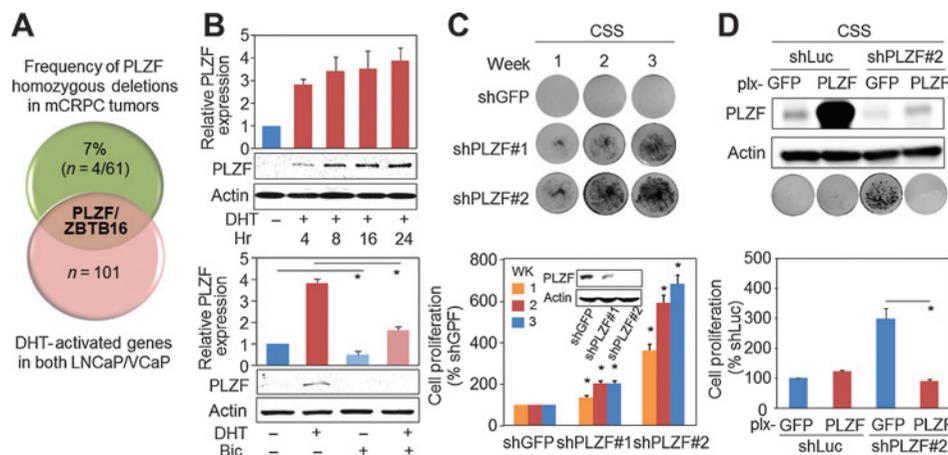

Figure 1.
PLZF is an androgen-regulated gene involved in growth suppression. A, Venn diagram showing the frequency (%) of PLZF homozygous deletions (n = homozygous deletions/total mCRPC tumors; ref. 11) and PLZF as a putative tumor suppressor gene with strongest AR binding merged from two AR cistrome datasets. B, qRT-PCR and Western blotting were used to measure PLZF mRNA and protein expression of LNCaP cells, which were cultured in CSS, followed by 10 nmol/L of DHT and/or 10 μmol/L of bicalutamide (Bic) treatment. The colonies were stained by CV and photographed. C and D, the efficiency and efficacy of PLZF shRNA knockdown (C) and ectopic re-expression of PLZF (D) were measured by Western blot. Each column was relative to the corresponding first column and shown as mean ± SD ($n \geq 3$). *, $P < 0.05$.
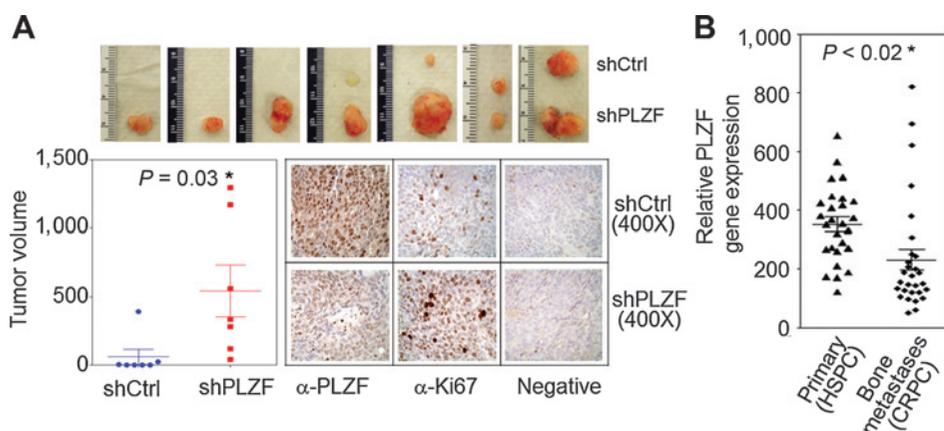
Figure 2.
PLZF functions as a tumor suppressor *in vivo*. A, tumor formation assays of castrated male nude mice injected with shCtrl and PLZF stable silencing 22Rv1 cells. Bottom right, averaged xenograft tumors (mean ± SEM); left, PLZF and Ki67 immunohistochemistry were used to monitor the efficacy of PLZF knockdown and cell proliferation in 22Rv1 xenografts. B, PLZF gene expression from 27 hormone-sensitive prostate cancers (HSPC) and 29 bone mCRPCs.

Prostate Cancer Foundation (SU2C/PCF), which will be part of a larger SU2C genomic landscape article (personal communications). This prompted us to explore the role of PLZF in prostate cancer. Here, we postulated that because PLZF was androgen regulated, AR might activate PLZF, an intermediate tumor suppressor gene that might derepress an oncogenic program with androgen depletion. This was based on the observation that ADT induces the expression of androgen-repressed genes that normally regulate androgen synthesis, DNA replication, and cell cycle progression in CRPC models (12).

Taking an agnostic approach, we sought androgen-regulated candidate tumor suppressor genes. We compiled two AR cistrome datasets and showed that PLZF was the canonical tumor suppressor gene with strongest androgen-induced AR recruitment to its putative enhancer regions in two androgen-dependent prostate cancer cell lines, LNCaP and VCaP (Supplementary Figs. S1 and S2A), implying that the tumor suppression function of PLZF may be diminished upon ADT treatment.

The androgen-stimulated effect on PLZF expression was demonstrated in LNCaP, VCaP, and 22Rv1 cells (Fig. 1B; Supplementary Fig. S2B and S2C). More importantly, PLZF expression was repressed by an antiandrogen (bicalutamide) in LNCaP cells (Fig. 1B, bottom).

To explore the tumor suppressive function of PLZF on prostate cancer, we examined the biologic effect of altered PLZF expression on cell growth in androgen-depleted condition. Knockdown of PLZF using four different shRNA constructs (shPLZF#1~#4) promoted androgen-independent growth in LNCaP cells (Fig. 1C; Supplementary Fig. S3A). Conversely, re-expression of PLZF reversed the androgen-independent growth mediated by PLZF depletion (Fig. 1D).

To determine whether PLZF perturbation might promote androgen-independent growth *in vivo*, we also analyzed the growth of 22Rv1 prostate cancer xenograft expressing PLZF shRNAs, in castrated nude mice. Consistent with the *in vitro* observations, PLZF knockdown enhanced tumor formation in castrate levels of androgen (Fig. 2A). Altogether, our results show that PLZF is a putative AR-regulated tumor suppressor gene.

Next, we interrogated the extent to which PLZF expression might be altered in patient-derived CRPC tumor samples and found that mean PLZF expression was significantly lower in CRPC bone metastases compared with primary tumors (Fig. 2B),
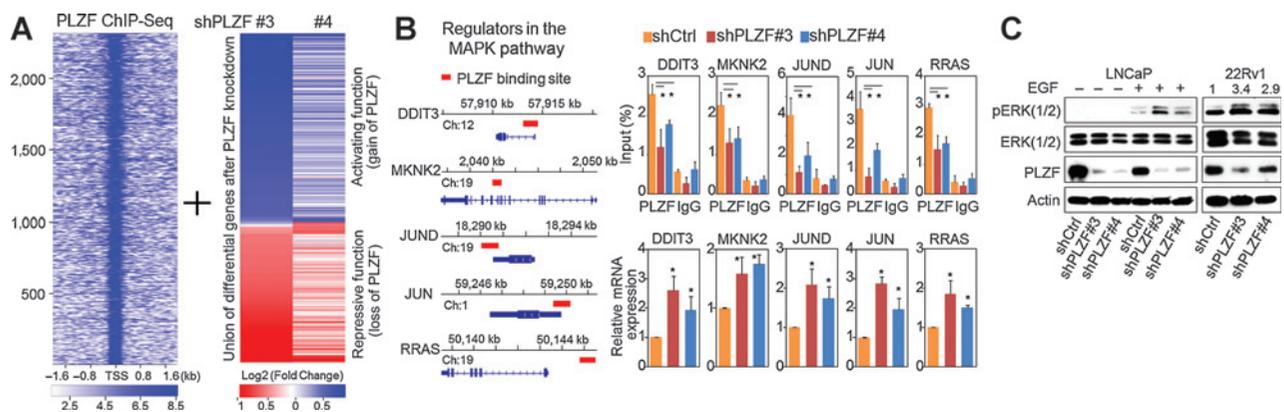

Figure 3.
Bioinformatic analysis of the PLZF transcriptional program. A, heat maps of PLZF ChIP-seq signal ±2.0 kb around the PLZF peak summit in LNCaP. The color scale indicates average signal. The numbered index of PLZF peaks is shown to the left. A cluster of differentially expressed genes in the LNCaP with stable knockdown of shPLZF# 3 or 4. B, KEGG pathway analysis of PLZF-repressed genes. PLZF direct targets are highlighted in bold red. C, Left, schematic graph shows the PLZF-binding sites (red bars) within the PLZF target gene loci as defined by PLZF. Right, ChIP-seq in LNCaP cells. ChIP and qRT-PCR validation of PLZF binding and gene expression to selected PLZF targets. Values were the mean ± SD ($n \geq 3$); *, $P < 0.05$. D, Western blot of LNCaP and 22Rv1 was used to measure PLZF expression and ERK1/2 activity with or without EGF (10 ng/mL) stimulation.

consistent with the notion that ADT suppresses serum and tissue androgens and results in diminished AR pathway activity (9).

The subcellular localization of PLZF is mainly in the nucleus where it achieves its transcriptional repression by binding to the regulatory elements in the promoter region of the target genes (7). In order to uncover PLZF-regulated transcriptome, we defined the PLZF cistrome using PLZF ChIP-seq and gene profiling data sets (Fig. 3A; Supplementary Tables S1 and S2). Next, we investigated the potential biologic consequence of PLZF suppression. Genes whose expression was upregulated in PLZF-depleted LNCaP cells were subjected to bioinformatic pathway analysis. KEGG analysis revealed that PLZF-repressed genes were significantly enriched in the MAPK signaling pathway, including 5 genes with PLZF-binding sites, *RRAS*, *MKNK2*, *DDIT3*, *JUND*, and *JUN* (Supplementary Table S3). ChIP- and qRT-PCR confirmed that these genes are part of the PLZF-repressed cistrome (Fig. 3B). More importantly, PLZF knockdown substantially induced phospho-ERK1/2 expression upon EGF stimulation in LNCaP (Fig. 3C, left). We also observed elevated levels of phospho-ERK activity in PLZF-depleted 22Rv1 cells (Fig. 3C, right). The inhibitory effect of MAPK inhibitors (UO126 and AZD6244) on PLZF-depleted LNCaP was assessed. Cells with PLZF depletion responded better to MAPK inhibitors as compared with shCtrl, implying that the MAPK pathway may be activated due to loss of PLZF expression (Supplementary Fig. S4).

Although our results suggest that PLZF regulates ERK1/2 activity, it is unlikely that the mechanism underlying ERK activation only depends on PLZF transcriptional modulation. Moreover, PLZF has been shown to regulate a variety of downstream targets at the posttranslational level (13). PLZF-regulated intracellular signaling molecules may also cross-talk with other regulatory pathways. Nonetheless, taken together, our data suggest that suppression of PLZF may permit sustained prostate cancer cell growth under conditions of androgen deprivation in part by de-repressing key tumorigenic mechanisms, such as ERK1/2 signaling. This finding may partly explain

and is in agreement with previous findings that MAPK signaling is upregulated in some CRPC murine models and patient-derived tumor samples (14–16). Thus, in the subset of patients with low PLZF expression including genetic loss, MAPK pathway inhibition may be of particular importance.

To begin to explore the potential effect of PLZF loss on AR-targeted therapy, we evaluated the impact of enzalutamide on the growth of prostate cancer cells in the absence or presence of PLZF knockdown. As expected, enzalutamide completely killed the shLuc-silenced cells when cultured in the regular conditioned medium. PLZF-depleted cells showed the ability to grow, although to a lesser extent, even in presence of enzalutamide (Fig. 4A). When we conducted the same experiment culturing the cells in androgen-deprived medium (charcoal-stripped serum, CSS), we observed a similar growth pattern. While shLuc cells remained quiescent in absence of androgens, shPLZF cells showed slight sensitivity to enzalutamide at early time point, becoming progressively resistant to the drug at late time points (Fig. 4B). To determine whether the presence of AR is required for PLZF-dependent growth, we introduced an Isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible shAR in the shPLZF-stable LNCaP cells. Strikingly, our data showed that PLZF loss enables LNCaP cells to proliferate even in the absence of androgens or AR expression (Fig. 4C). These results imply that PLZF inactivation may be a key factor in the development of resistance to AR-directed therapeutics, such as enzalutamide. Collectively, our data suggest that PLZF suppression or genetic loss may underlie a novel mode of resistance to ADT, wherein an AR-repressed oncogenic program facilitates residual prostate tumor cells to adjust to castrate levels of androgens to survive or grow.

In view of the AR-dependent mechanisms for CRPC development, ADT may directly or indirectly activate an AR-repressed network, although we cannot completely exclude the involvement of oncogenic activation mediated by persistent AR expression in residual prostate tumors. Accordingly, we report that the upregulation of the PLZF-repressed oncogenic program is an acquired mechanism in response to ADT and that genetic loss of
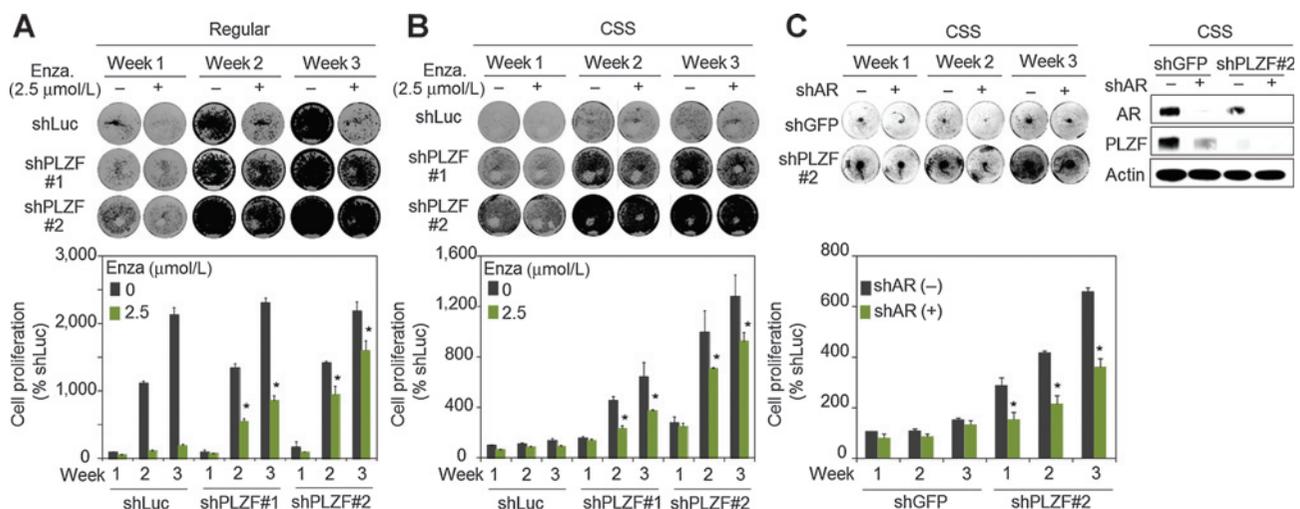
Figure 4.
PLZF depletion alters the growth-inhibitory effect of enzalutamide. LNCaP cells with or without PLZF knockdown were cultured in 5% FBS (A) or CSS medium (B) and treated with or without 2.5 μmol/L of enzalutamide or IPTG-inducible shAR knockdown (C), followed by CV staining at the time as indicated. Each column was relative to the corresponding first column and shown as mean ± SD ($n \geq 3$); *, $P < 0.05$.

PLZF in the course of disease is important molecular event in the emergence of CRPC and development of resistance to ADT and perhaps enzalutamide. Presumably, prostate cancer genomic and transcriptomic information may permit better molecular classification and provide new insights into the mechanisms of resistance to androgen/AR signaling blockade, thus aiding the design of future therapeutic combinations to overcome drug resistance.

Disclosure of Potential Conflicts of Interest

M. Brown reports receiving a commercial research grant and is a consultant/advisory board member for Novartis. L.A Garraway reports receiving a commercial research grant from Novartis, has ownership interest (including patents) in Foundation Medicine, and is a consultant/advisory board member for Boehringer Ingelheim, Foundation Medicine, and Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: C.-L. Hsieh, S. Gao, C. Cai, C.J. Sweeney, M. Brown, L.A. Garraway, P.W. Kantoff
Development of methodology: C.-L. Hsieh, G. Botta, E.M. Van Allen, L.A. Garraway, P.W. Kantoff
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-L. Hsieh, G. Botta, E.M. Van Allen, L.A. Garraway, P.W. Kantoff
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.-L. Hsieh, G. Botta, S. Gao, T. Li, E.M. Van Allen, C. Cai, H.H. He, C.J. Sweeney, M. Brown, L.A. Garraway, P.W. Kantoff

Writing, review, and/or revision of the manuscript: C.-L. Hsieh, G. Botta, S. Gao, E.M. Van Allen, C.J. Sweeney, M. Brown, S.P. Balk, P.S. Nelson, L.A. Garraway, P.W. Kantoff
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-L. Hsieh, G. Botta, E.M. Van Allen
Study supervision: C.-L. Hsieh, L.A. Garraway, P.W. Kantoff

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