Detection of Androgen Receptor Mutations in Circulating Tumor Cells in Castration-Resistant Prostate Cancer

Yuqiu Jiang,1* John F. Palma,1 David B. Agus,2† Yixin Wang,1 and Mitchell E. Gross2†

1 Veridex, LLC, a Johnson & Johnson company, San Diego, CA; 2 Louis Warschaw Prostate Cancer Center, Cedars-Sinai Medical Center, Los Angeles, CA; * address correspondence to this author at: Veridex, LLC, a Johnson & Johnson company, 1001 US Route 202 North, Raritan, NJ 08869. Fax 908-704-3882; e-mail jjiang2@its.jnj.com. † Current address: Westside Prostate Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA.

BACKGROUND: Coding mutations in the AR (androgen receptor) gene have been identified in tissue samples from patients with advanced prostate cancer and represent a possible mechanism underlying the development of castration-resistant prostate cancer (CRPC). There is a paucity of tumor-derived tissue available for molecular studies of CRPC patients. Circulating tumor cells (CTCs) in the blood of CRPC patients represent a possible avenue for interrogating the disease of such patients.

METHODS: Circulating tumor cells were captured with the CellSearch® Circulating Tumor Cell (CTC) Kit and with the CellSearch Profile Kit plus Qiagen’s AllPrep DNA/RNA Micro Kit for the measurement of the CTC count per 7.5 mL of blood and for the isolation of nucleic acids, respectively. The AR gene was amplified by the PCR, and mutation status and relative abundance were analyzed by applying Transgenomic’s WAVE® denaturing HPLC technology followed by direct sequencing.

RESULTS: AR mutations were detected in 20 of 35 CRPC patients; 19 missense mutations, 2 silent mutations, 5 deletions, and 1 insertion were observed. The relative abundance of the mutants in the amplified products ranged from 5% to 50%. Many of the AR mutations were identified in surgical biopsies or at autopsy and were associated with resistance to androgen-directed therapies.

CONCLUSIONS: AR mutations can be identified in CTC-enriched peripheral blood samples from CRPC patients. This approach has the potential to open new perspectives in understanding CTCs and the mechanisms for tumor progression and metastasis in CRPC.

The androgen receptor (AR)3 plays a key role in prostate function and the development of prostate cancer (1–3). Nearly all patients with recurrent prostate cancer respond to androgen-deprivation therapy; however, some patients develop a progressive and potentially lethal form of prostate cancer despite low concentrations of androgens after castration. Treatment-emergent mutations in the AR4 (androgen receptor) gene have been identified in many patients with advanced prostate cancer and are a mechanism of acquired resistance to androgen-deprivation therapy (4). In general, however, no high-quality tissue samples are available for molecular analyses in patients with castration-resistant prostate cancer (CRPC). Most patients have a biopsy performed only at the time of diagnosis (or at surgery), which represents castration-sensitive prostate cancer sampled many years before the actual development of CRPC. Because of this lack of access to human tissue samples at the CRPC stage, the molecular and cellular changes that underlie the development of CRPC in prostate cancer consequently have been studied intensively only with cellular and animal models. There is a critical need to develop noninvasive technologies to study molecular events in CRPC.

The CellSearch® Circulating Tumor Cell (CTC) Kit (Veridex), the first in vitro diagnostic test using CTC technology cleared by the US Food and Drug Administration, has been used for prognostic and disease monitoring in patients with metastatic breast, colorectal, and prostate cancers, and offers a potential noninvasive method for tumor analysis (5–7). To better understand the molecular mechanisms of response to therapy and drug resistance in patients, we sought to expand the utility of the technology to directly interrogate molecular markers in CTCs. We report a method for detecting coding AR mutations in peripheral blood samples that is based on CTC enrichment followed by analysis with a high-resolution mutation-detection system.

Colorectal tissue samples representing cancer and healthy tissue were used in a titration experiment to determine the detection capability of Transgenomic’s WAVE® denaturing HPLC (DHPLC) technology (8). The frequencies of the KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) G12V mutation in the tumor and nonpathologic samples were 50% and 0%, re-

3 Nonstandard abbreviations: AR, androgen receptor; CRPC, castration-resistant prostate cancer; CTC, circulating tumor cell; DHPLC, denaturing HPLC.
4 Human genes: AR, androgen receptor; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog.
spectively (confirmed by sequencing). A series of samples containing 25%, 6.25%, 2.5%, and 1% of the KRAS G12V mutant was generated by mixing these 2 colorectal samples. Two tubes each containing 7.5 mL blood were collected from 40 patients at a single institution under an institutional review board–approved protocol and after informed consent was obtained. All patients had metastatic CRPC before receiving first-line or salvage treatment with cytotoxic chemotherapy (see Table 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue9). The blood samples were processed for enumeration (CellSave; Veridex) or molecular characterization (EDTA) with the CellSearch system according to the manufacturer’s standard protocol.

Nucleic acids were extracted from the isolated CTCs (EDTA tube) with the Qiagen AllPrep RNA/DNA Micro Kit. Pairs of primers flanking KRAS exon 2 were used for amplification. CRPC samples were analyzed with primers for 3 amplicons designed to amplify AR exons 3–8, with each amplicon spanning 2 consecutive exons. cDNA was synthesized according to the oligo(dT) protocol of the SuperScript® III First-Strand Synthesis System for RT-PCR kit (Invitrogen). PCR amplification was performed according to Transgenomic’s standard protocol.

The SURVEYOR Nuclease (Transgenomic) digestion reaction was performed according to the manufacturer’s recommendations with KRAS exon 2 as a control and with 3 AR amplicons to scan for heterozygous mutations with SURVEYOR Nuclease. DNA fragments were first analyzed by HPLC under nondenaturing conditions with the Transgenomic WAVE® System Model 4500HT-HS. Fragment sizes were measured with the aid of the Transgenomic Sizing Standard (9). We sequenced all amplicons bidirectionally with the 3730 DNA Analyzer (Applied Biosystems) to identify homozygous mutations as well as to confirm heterozygous mutations identified by SURVEYOR Nuclease digestion. For detailed descriptions of the experimental procedures, see Supplemental Data File 1 in the online Data Supplement.

In the CTC-enriched samples, we anticipated a large representation of the wild-type AR sequence, which is derived from both prostate cancer cells and nonpathologic leukocytes with the wild-type AR gene. Therefore, we performed a titration experiment to investigate the detection capability of the SURVEYOR/WAVE method. We demonstrated that the WAVE DHPLC technology is capable of detecting mutant species at relative abundances as low as 2.5% in a tumor sample (see Supplemental Fig. 1 in the online Data Supplement).

Next, we sought to combine CellSearch enrichment with the SURVEYOR/WAVE sequencing approach to identify AR mutations in the peripheral blood of CRPC patients. Fig. 1 illustrates the process flow for this method. Forty patients were enrolled in the study, and AR sequence analysis was performed for 35 of these individuals. For the 5 disqualified individuals, we identified either insufficient quantities of RNA in the CTC-enriched samples for sequencing or logistical issues, such as underfilled tubes or samples received >36 h after collection.

We identified a total of 27 AR mutations in 20 (57%) of the 35 individuals who could be evaluated (Table 1). Most of the mutations represented coding mutations, which were classified as missense (n = 19), deletions (n = 5), insertions (n = 1), and silent mutations (n = 2). Many of the mutations had previously been described as acquired mutations in tumors from CRPC patients. Five of the mutations identified (W741R, V757A, R846G, H874Y, and T877A) have been characterized previously as somatic mutations in metastatic prostate cancer tissue (10). Many of the patients exhibited multiple AR mutant forms. For example, patient 13 had both H874Y and T877A, each at a
relative abundance of 10%. We also noted the occurrence of the W741L mutation in patient 34, who had extensive prior treatment with a nonsteroidal androgen, bicalutamide. Hara et al. have shown with experiments with a subline of LNCaP cells that the W741L mutation may be a possible mechanism causing bicalutamide withdrawal syndrome (11). The performance of the assay is exemplified for the patients in whom the relative abundance of the mutant species approached the limit of detection, which we estimated to be 2.5% (see Supplemental Fig. 1 in the online Data Supplement). For example, we detected the W741L mutation at a relative abundance of only about 5% in sample 34b from patient 34 (Table 1).

Our data also support the general consistency of this method. Two samples were collected from patient 34 (34a and 34b, before and after docetaxel chemotherapy, respectively). This patient had 1378 CTCs (34a) before starting docetaxel therapy; the CTC number had decreased to 278 (34b) by 3 months after treatment. The W741L mutation, however, was detected in this patient’s AR gene in both samples. The frequency of this mutant form was 50% before treatment and 5% three months later.

Although many of the mutations have previously been described (http://androgendb.mcgill.ca/map.gif), we also found new AR mutations in these CRPC patients. The most commonly observed mutation in our sample set was the G589_A628 deletion (n = 4) encoded in exon 3, which is predicted to disrupt the second zinc finger domain of AR. The G589_A628 deletion in the AR protein was previously reported in breast cancer patients as due to an alternative splice variant of the AR gene (12). Mutations affecting the same region of the DNA-binding domain have also been implicated in androgen-insensitivity syndromes and prostate cancer (13, 14). To our knowledge, this result is the first time this AR splice variant has been found in prostate cancer. In addition, we detected another novel AR internal deletion (H663_A680) in a single patient (patient 21). This deletion is in exon 4 and may involve the ligand-binding domain. Additional studies are needed to determine the functional significance of these variants in the growth of androgen-independent cancers.

The genetic heterogeneity of CTCs in a high background concentration of healthy leukocytes have limited the applications of sequencing technologies for tu-

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>CTCs, n</th>
<th>Missense</th>
<th>Insertion</th>
<th>Silent</th>
<th>Deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
<td>G589_A628 del/20%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>22</td>
<td>M780V/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>17</td>
<td>G743E/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>H874Y/50%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>690</td>
<td>H874Y/10%; T877A/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>2362</td>
<td>H874Y/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>R846G/15%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>Y763N/5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>M775V/10%; L790P/5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>37</td>
<td>V757A/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>7</td>
<td>E642G/30%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>36</td>
<td>V769V/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>7</td>
<td>A687A/30%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>594</td>
<td>G589_A628 del/10%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>0</td>
<td>M895V/15%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>5</td>
<td>Y781H/5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34a</td>
<td>1378</td>
<td>W741L/50%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>34b</td>
<td>282</td>
<td>W741L/5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>108</td>
<td>V769G/10%; I815T/5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>59</td>
<td>W741R/5%; F916L/15%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. AR mutations detected in isolated CTC samples from CRPC patients.
malignant samples obtained from peripheral blood. The SURVEYOR/WAVE DHPLC technology is a sensitive method for detecting gene mutations that are present at very low frequencies in heterogeneous samples (9). The technology has been developed and applied as a high-sensitivity, lower-cost alternative to direct sequencing of cancer tissue samples (9). Given that the CellSearch system is expected to achieve an enrichment of up to 4 orders of magnitude with a capture efficiency of >85% (15), we are not surprised to have demonstrated that the CellSearch enrichment technology is capable of detecting mutations in CTCs.

In all cases that we examined, AR mutant isoforms represent a fraction (range, 5%–70%) of all species observed. We also note that the mutant fraction does not directly correlate with the CTC count, in that mutations can be observed in patients with a very low number of cells or even no observed CTCs. Stringent morphologic criteria, including the finding of a pancytokeratin-positive, CD45-negative cell structure with a retained nucleus (as assessed by 4',6-diamidino-2-phenylindole staining), are used to define a CTC with CellSearch. In contrast, the mutation data are collected via the use of specific primers designed to amplify epithelial cell adhesion molecule–enriched RNA, which is derived from either CTCs or free nucleic acids. Therefore, enumeration and sequencing could provide complementary information on patients’ CTCs.

Our method opens new avenues for the molecular interrogation of gene mutations in CTCs isolated from patients’ blood samples. We have demonstrated a noninvasive method for unbiased detection of multiple AR mutations in CRPC patients. In addition, our approach may enable real-time sampling for the exploration of cellular and molecular characteristics of metastatic cancer, and it may offer clinical and practical advantages over research approaches that rely on sequential imaging or biopsies of patients with metastatic cancer. The understanding of the molecular features of CTCs may help physicians select and direct specific treatments for individual cancer patients.

References


Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: Y. Jiang, Johnson & Johnson; J.F. Palma, Veridex, a Johnson & Johnson company; Y. Wang, Johnson & Johnson.
Consultant or Advisory Role: None declared.
Honoria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank the patients who participated in the study.

Previously published online at DOI: 10.1373/clinchem.2010.143297

Clinical Chemistry 56:9 (2010) 1495