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High-Resolution Genomic Profiling of Disseminated Tumor Cells in Prostate Cancer

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Circulating tumor cells and disseminated tumor cells (DTCs) are of great interest because they provide a minimally invasive window for assessing aspects of cancer biology, including tumor heterogeneity, a means to discover biomarkers of disease behavior, and a way to identify and prioritize therapeutic targets in the emerging era of precision oncology. However, the rarity of circulating tumor cells and DTCs poses a substantial challenge to the consistent success in analyzing their molecular features, including genomic aberrations. Herein, we describe optimized and robust methods to reproducibly detect genomic copy number alterations in samples of 2 to 40 cells after whole-genome amplification with the use of a high-resolution single-nuclear polymorphism-array platform and refined computational algorithms. We have determined the limit of detection for heterogeneity within a sample as 50% and also demonstrated success in analyzing single cells. We validated the genes in genomic regions that are frequently amplified or deleted by real-time quantitative PCR and nCounter copy number quantification. We further applied these methods to DTCs isolated from individuals with advanced prostate cancer to confirm their highly aberrant nature. We compared copy number alterations of DTCs with matched metastatic tumors isolated from the same individual to gain biological insight. These developments provide high-resolution genomic profiling of single and rare cell populations and should be applicable to a wide-range of sample sources. *(J Mol Diagn 2016, 18: 131—143; http://dx.doi.org/10.1016/j.jmoldx.2015.08.004)*

Tumor heterogeneity complicates our understanding of the biological mechanism of cancer and presents challenges for effective diagnostic and therapeutic approaches in the clinic. Among techniques that help define the alterations present in all tumor subpopulations, methods to detect and isolate tumor cells from the blood or bone marrow of cancer patients provide new and relatively noninvasive alternatives for sampling solid tumors and are referred to as liquid biopsies.[1] Circulating tumor cells (CTCs) are shed from tumors into the blood and provide an index of the current tumor burden.[2] Although the half-life of a CTC is short (approximately 2 hours),[3] disseminated tumor cells (DTCs) have effectively migrated to the bone marrow, where they may remain dormant for up to several decades but may gain metastatic potential eventually.[4] Quantification of both CTCs and DTCs has shown promise as novel diagnostics to measure tumor burden and to assess the risk of relapse after initial therapies in breast and prostate cancers.[5–7] A recent meta-analysis of 33 clinical studies supported the prognostic value of CTCs/DTCs for survival outcome in prostate cancer.[8] Molecular characterization of CTCs and DTCs will provide information about genomic aberrations, expression profiles, or other cellular perturbations that may better predict treatment response or disease outcome.

The rarity of CTC/DTCs poses a substantial challenge to the consistent success in analyzing the genome of these

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cells. Previous studies were limited to low-resolution analyses of a subset of genes or genomic loci.[9] Recent advances in the genome-wide analysis of single cells with next-generation sequencing approaches have offered great promise in the clinical application of CTC/DTCs as prognostic and predictive biomarkers, especially focused on somatic mutations.[10,11] Nevertheless, the few whole-genome profiling studies that examine somatic copy number aberrations (SCNAs) published thus far used relatively low-resolution bacterial artificial chromosome-based or oligonucleotide-based comparative genome hybridization techniques.[12–15] A robust, reproducible, cost-effective, and high-resolution genomic profiling method is sorely desired by many researchers.

Here, we describe in detail an optimized method for consistent and robust genome-wide profiling of prostate cancer DTCs that yields high resolution for SCNAs on samples of 2 to 40 cells from individual patients. With the use of well-characterized cell lines, we have optimized methods for the whole-genome amplification (WGA) and single-nuclear polymorphism (SNP) array analysis of archived samples. We refined computational methods to reduce noise and to improve the segmentation and copy number (CN)-calling methods for data generated from WGA samples. We then applied these methods to DTCs isolated from patients with advanced prostate cancer to confirm the highly aberrant nature of these cells and to compare SCNAs with those in matched metastatic tumors from the same individual. We validated genes in genomic regions that are frequently amplified or deleted with real-time quantitative PCR and nCounter CN quantification. These developments provide high-resolution genomic profiling of single and rare cell populations and should be applicable to a wide-range of sample sources, including CTCs, formalin-fixed, paraffin-embedded-derived cells, and embryonic cell testing.

Materials and Methods

Cell Lines and Patient Samples

The LNCaP prostate adenocarcinoma cell line was maintained according to ATCC (Manassas, VA) instructions. A male velocardiofacial (VCF) syndrome cell line GM07939B [46,XY,del(22)(q11.21q11.22)] was obtained from the Coriell Institute (Camden, NJ) and cultured according to the provided protocols. Ten normal female lymphoblast reference (NFR) lines were a gift from Dr. Barbara Trask (Lawrence Livermore National Laboratory, Livermore, CA) and were previously described.[16] All samples that contained $\leq$40 cells were collected with a micromanipulator as previously described.[12] Bulk genomic DNA from cell lines was extracted from cell pellet that contained approximately 2×10^6 cells, using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA).

Metastatic tumor samples from prostate cancer patients were collected at the University of Washington from autopsies performed within 6 hours of death under the aegis of the rapid autopsy program.[17] All tissues were frozen immediately and stored at −80°C. Hematoxylin and eosin-stained tissue sections were reviewed by a pathologist for verification of histology. This study was approved by the institutional review board at the Fred Hutchinson Cancer Research Center and University of Washington. The data discussed in this publication were deposited in National Center for Biotechnology Information's Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE73180).

Isolation of DTCs

The methods to isolate DTCs from bone marrow aspirates were described previously.[6,18] Briefly, 10 mL of bone marrow was aspirated from the right iliac crest and combined with 10 mL of 6% sodium citrate. Samples were processed as soon as possible. Aspirates were placed over 15 mL of Ficoll-Isopaque 1.077 g/mL (Accurate Chemical, Westbury, NY) and centrifuged to yield a mononuclear cell layer. Two rounds of immunomagnetic selection (Miltenyi Biotec, San Diego, CA) were used to enrich for DTCs. First, negative selection of leukocytes, megakaryocytes, and platelets was performed with antibodies to CD45 and CD61. Second, immunomagnetic beads coated with an anti–epithelial cell adhesion molecule (EpCAM) antibody (clone HEA-125/CD326) were used to positively select cells that expressed epithelial antigens. The eluted fraction contained a cell mixture enriched for cells of epithelial origin.

To identify and isolate DTCs, the enriched cell population was immunostained with fluorescein isothiocyanate-labeled BerEP4 clone of the anti-EpCAM antibodies (Dako, Carpinteria, CA), which bind to a different epitope on human EpCAM than the antibody used for positive selection.[6] A phycoerythrin-conjugated anti-CD45 antibody was also used to counterstain leukocytes. Cells were viewed under fluorescent light with the use of an inverted microscope. DTCs were defined as $CD45^-$ cells that have intermediate-to-high labeling with anti-EpCAM. $EpCAM^+/CD45^-$ cells were kept on ice and viewed under fluorescent light, and intact cells with a clearly defined EpCAM-labeled membrane were isolated with a micromanipulator and were dispensed into individual snap-top PCR tubes that contained 10 μL of water. Samples were frozen immediately on dry ice and stored at −80°C until WGA. We were able to generate data of comparable quality from samples stored up to 12 years relative to DTCs acquired within the past 2 years.

DNA Extraction from Bone Metastases and Laser-Captured Materials

Bone, lymph node, and liver metastases were laser capture microdissected with an Arcturus Veritas instrument and collected on CapSure Macro LCM Caps (Life Technologies, Carlsbad, CA). DNA was isolated with QiaAMP DNA Blood Micro kit (Qiagen) according to manufacturer's protocol with the following modifications: samples were incubated with 15 μL buffer ATL and 10 μL proteinase K (Life Technologies, Carlsbad, CA) for 14 to 20 hours at