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Assessing Copy Number Alterations in Targeted, Amplicon-Based Next-Generation Sequencing Data

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Changes in gene copy number are important in the setting of precision medicine. Recent studies have established that copy number alterations (CNAs) can be detected in sequencing libraries prepared by hybridization-capture, but there has been comparatively little attention given to CNA assessment in amplicon-based libraries prepared by PCR. In this study, we developed an algorithm for detecting CNAs in amplicon-based sequencing data. CNAs determined from the algorithm mirrored those from a hybridization-capture library. In addition, analysis of 14 pairs of matched normal and breast carcinoma tissues revealed that sequence data pooled from normal samples could be substituted for a matched normal tissue without affecting the detection of clinically relevant CNAs (>|2| copies). Comparison of CNAs identified by array comparative genomic hybridization and amplicon-based libraries across 10 breast carcinoma samples showed an excellent correlation. The CNA algorithm also compared favorably with fluorescence *in situ* hybridization, with agreement in 33 of 38 assessments across four different genes. Factors that influenced the detection of CNAs included the number of amplicons per gene, the average read depth, and, most important, the proportion of tumor within the sample. Our results show that CNAs can be identified in amplicon-based targeted sequencing data, and that their detection can be optimized by ensuring adequate tumor content and read coverage. *(J Mol Diagn 2015, 17: 53—63; http://dx.doi.org/10.1016/j.jmoldx.2014.09.008)*

The identification of molecular aberrations present in a tumor sample is becoming important in delivering precision cancer care. Targeted sequencing using next-generation technologies is effective in identifying the single-nucleotide substitutions and short indels that may help guide treatment decisions.[1–9] Two widely used enrichment strategies for targeted sequencing are hybridization-capture, in which oligonucleotide baits complementary to the regions of interest are hybridized with fragmented genomic DNA,[3] and PCR, in which a pool of primers is used to generate target-specific amplicons.[4,10] Both of these approaches work well on DNA purified from formalin-fixed, paraffin-embedded (FFPE) tumor tissue, and they require only small amounts of input DNA (10 to 100 ng).

Copy number alterations (CNAs) are also important in personalized cancer diagnostics. *ERBB2* amplification is routinely screened in breast carcinomas to determine whether HER2-targeted therapies should be included in a patient's treatment plan. Similarly, amplifications of *FGFR1*, *EGFR*, *MET*, and *PIK3CA* are all being targeted in ongoing clinical trials. There are a variety of technologies that can be used to measure CNAs in tumor DNA, including genome-wide approaches such as array comparative genomic hybridization (aCGH) and whole-genome sequencing, as well as targeted approaches, such as whole-exome sequencing, single-nucleotide polymorphism (SNP) arrays, quantitative PCR, and fluorescence *in situ* hybridization (FISH).[11–17] Among these methods, those based on next-generation sequencing

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(NGS) are gaining in popularity, because information on CNAs can be derived from the same data used to detect sequence alterations. Algorithms for assessing CNAs have been developed for NGS protocols that are based on hybridization-capture, whether in the setting of whole-exome sequencing[12] or targeted sequencing.[1,3,5,8] In contrast, little work has been done on CNA assessment in NGS data from amplicon-based libraries.

Herein, we developed and validated an algorithm for assessing CNAs in NGS data derived from amplicon-based libraries of FFPE tumor DNA. We compared the results with CNAs assessed in a hybrid-capture library, as well as with CNAs determined from aCGH data, and from FISH for specific genes. In addition, we systematically examined several factors that can influence CNA detection, including tumor purity, the number of amplicons per gene, and the number of reads per amplicon. Our results show that CNAs are readily detected in amplicon-based libraries and correlate well with other methods. However, the sensitivity for CNAs is influenced by several parameters that should be taken into account in both the design of targeted panels and the interpretation of the NGS data that they yield.

Materials and Methods

Tumor Specimens and DNA Preparation

This study was conducted in accordance with federal and institutional guidelines. For all samples, excluding WA25 (see below), blocks of FFPE tumor or unstained sections of FFPE tissue were obtained from the pathology archives of Oregon Health and Science University (Portland, OR). The diagnosis in each case was confirmed by a board-certified pathologist (C.L.C.). Tumor-rich areas (20% to 90%) were macrodissected from unstained sections (5 μm thick) by comparison with a hematoxylin and eosin (H&E)−stained slide, and genomic DNA was extracted using a Macherey-Nagel NucleoSpin Tissue Kit (Clontech, Mountain View, CA). For 14 of the breast tumor samples, morphologically normal areas were identified and used as a source for matched normal DNA; these samples also served in the generation of a pool of data from normal DNA. Genomic DNA (20 ng) was used for library preparations from the tumor samples and from the matched normal samples.

Preparation of Amplicon Libraries

A custom Ion AmpliSeq (Ion Torrent, Carlsbad, CA) solid tumor panel was used to generate target amplicon libraries. This panel covers some or all of the coding exons of 37 genes known to play a role in cancer: *AKT1*, *AKT2*, *AKT3*, *ALK*, *BRAF*, *CDK4*, *CDKN2A*, *DDR2*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *GNA11*, *GNAQ*, *GNAS*, *KDR*, *KIT*, *KRAS*, *MAP2K1*, *MET*, *HRAS*, *NF1*, *NOTCH1*, *NRAS*, *NTRK2*, *NTRK3*, *PIK3CA*, *PIK3R1*, *PTEN*, *RAC1*, *RB1*, *RET*, *STK11*, *TSC1*, *TSC2*, *TP53*, and *VHL*. The number of amplicons per gene in the panel varies from 1 to 145. DNA derived from FFPE tissue (20 ng) was amplified by PCR using the premixed AmpliSeq primer pools and AmpliSeq HiFi master mix (Ion AmpliSeq kit version 2.0). Primer sequences were manufactured specifically for use with the Ion AmpliSeq kits and contained proprietary modifications. The resulting 1164 multiplexed amplicons were treated with FuPa reagent (Ion Torrent) to partially digest primer sequences and phosphorylate the amplicons. The amplicons were then ligated to Ion Xpress bar-coded adapters, according to the manufacturer's instructions (Ion Torrent). The Ion Library Quantitation Kit was used to determine the library concentration.

Emulsion PCR and Sequencing

Multiplexed bar-coded libraries were amplified for 20 cycles by emulsion PCR on Ion Sphere particles (ISPs) at a 1:2 ratio of total library molecules/ISPs (280×10^6 molecules per reaction) (Ion Xpress Template kit version 2.0; Ion Torrent). The templated ISPs were recovered from the emulsion, and the ratio of templated ISPs/empty ISPs was determined by a fluorometric assay using fluorescently labeled oligonucleotides complementary to adapter sequences. The optimal templated signal ratio was determined to be between 10% and 40%. Positive templated ISPs were biotinylated during the emulsion PCR process so that the samples with an optimal templated signal ratio were then enriched with Dynabeads MyOne streptavidin C1 beads (Life Technologies/Thermo Fisher, Carlsbad, CA). Eight bar-coded samples were multiplexed on an Ion 318 chip. Sequencing was performed on a Personal Genome Machine (PGM) sequencer (Ion Torrent) using the Ion PGM 200 sequencing kit 2.0, according to the manufacturer's instructions. Torrent Suite software version 4.0 (Ion Torrent) was used to parse bar-coded reads, to align reads to the reference genome, and to generate run metrics, including chip loading efficiency and total read counts and quality. The total reads per run and the average number of reads per amplicon are listed in Supplemental Table S1.

CNAs in Castration-Resistant Prostate Cancer Sample WA25

WA25 was obtained from a rapid autopsy performed at the University of Michigan Health Systems (Ann Arbor, MI) on a patient who died of castration-resistant prostate cancer. This sample was collected under prior informed consent of the patient and previous University of Michigan Institutional Review Board approval. H&E-stained sections from FFPE blocks were reviewed by a board-certified pathologist (S.A.T.), and a representative section with >50% tumor content and a benign tissue section were identified. Three sections (10 μm thick) were cut from each block, and the tumor sections were macrodissected to enrich tumor content.

For targeted sequencing, DNA was isolated using the Qiagen (Germantown, MD) Allprep FFPE DNA/RNA kit, according to the manufacturer's instructions, except with additional xylene/ethanol washes. DNA was quantified using the Qubit fluorometer (Life Technologies/Thermo Fisher). Bar-coded

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