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A Targeted High-Throughput Next-Generation Sequencing Panel for Clinical Screening of Mutations, Gene Amplifications, and Fusions in Solid Tumors

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Clinical next-generation sequencing (NGS) assay choice requires careful consideration of panel size, inclusion of appropriate markers, ability to detect multiple genomic aberration types, compatibility with low quality and quantity of nucleic acids, and work flow feasibility. Herein, in a high-volume clinical molecular diagnostic laboratory, we have validated a targeted high-multiplex PCR-based NGS panel (OncoMine Comprehensive Assay) coupled with high-throughput sequencing using Ion Proton sequencer for routine screening of solid tumors. The panel screens 143 genes using low amounts of formalin-fixed, paraffin-embedded DNA (20 ng) and RNA (10 ng). A large cohort of 121 tumor samples representing 13 tumor types and 6 cancer cell lines was used to assess the capability of the panel to detect 148 single-nucleotide variants, 49 insertions or deletions, 40 copy number aberrations, and a subset of gene fusions. High levels of analytic sensitivity and reproducibility and robust detection sensitivity were observed. Furthermore, we demonstrated the critical utility of sequencing paired normal tissues to improve the accuracy of detecting somatic mutations in a background of germline variants. We also validated use of the Ion Chef automated bead templating and chip loading system, which represents a major work flow improvement. In summary, we present data establishing the OncoMine Comprehensive Assay—Ion Proton platform to be well suited for implementation as a routine clinical NGS test for solid tumors. *(J Mol Diagn 2017, 19: 255—264; http://dx.doi.org/10.1016/j.jmoldx.2016.09.011)*

Next-generation sequencing (NGS) technologies are being established as platforms of choice for routine screening of tumor samples in a clinical setting.[1—4] In comparison with other sequencing platforms, the massively parallel sequencing capabilities of NGS provide a clear advantage by facilitating simultaneous screening of multiple markers in multiple samples for a variety of genomic aberrations using a single limited input of nucleic acid. For clinical applications, targeted sequencing of a limited set of clinically important genes has been the most practical approach. In the case of solid tumors, many laboratories have used relatively small pan-cancer panels, that screen approximately 50 (or fewer) genes, which interrogate prominent mutational hotspots only to maximize the use of sequencing capacity. This also helps to maintain reasonable costs and turnaround time, and minimize interpretation and reporting complexity.[5—8] However, this minimalistic approach results in several limitations, including lack of comprehensive screening of all known hotspots, insufficient sequencing of the gene backbone to glean copy number aberrations, and inability to screen the whole exome of tumor-suppressor genes, resulting in incomplete tumor mutation profiles. Consequently, there is a pressing need for implementing larger gene panels or sequencing the entire exome to

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increase sequencing coverage of a core set of clinically important genes and to incorporate additional genes of prognostic and predictive significance to facilitate screening a wider variety of tumor types.[9–11] However, relatively larger gene panels, which sequence hundreds of genes,[11,12] or whole exome sequencing[9] presents several challenges for high-volume routine clinical testing. These challenges include limited sequencing throughput as well as increasing the cost, turnaround time, and complexity of interpretation. A compromise solution, therefore, seems to be a balanced approach, which takes into consideration several factors, such as the difference in distribution and established functional significance of mutations in oncogenes and tumor suppressors, adequate gene backbone to facilitate higher detection confidence of copy number aberrations, and simultaneous detection of translocations or gene fusions to obtain comprehensive clinically useful information.

In this study, we tested the utility and clinically validated the OncoMine Comprehensive Assay (OCA), an extended pan-cancer NGS panel that takes into consideration many of the above mentioned design criteria for screening of formalin-fixed, paraffin-embedded (FFPE) solid tumor samples using a limited input of nucleic acids. This NGS panel screens 143 genes for mutations, and 49 of this gene set for copy number alterations (CNAs). This panel also has a RNA component, allowing screening for gene fusions involving 22 genes (Supplemental Table S1). The details regarding the development of this panel and its utility as a translational research tool have been published earlier[13]; however, thorough testing and validation of the OCA as a test for routine screening of clinically relevant genes in a clinical diagnostics laboratory is lacking. Herein, in a high-volume clinical molecular diagnostics laboratory, we have tested the utility of this panel for screening solid tumors. Using a large set of FFPE tissue specimens of different tumor types and variable cellularity, we have validated the PCR-amplicon based target capture by OCA panel and subsequent sequencing using the Ion Proton sequencer by establishing its analytical sensitivity, reproducibility, and limit of detection for various mutation types to establish it as a routine test for clinical sequencing of solid tumors in our laboratory.

Materials and Methods

Tumor Samples and Cell Lines

A study group of 121 tumors, including 24 with paired normal controls, and 6 cancer cell lines were used for sequencing studies. The cancer cell lines included H460 (HTB-177), SKBR3 (HTB-30), HL60 (CCL240), H2228 (CRL5935), and DLD1 (CCL221) from ATCC (Manassas, VA) and HCC78 (ACC 563) from DMSZ (Braunschweig, Germany). To mimic the FFPE tissue specimens, the cell lines were subjected to formalin fixation and embedded in paraffin before DNA extraction. The patient specimens included tumors from 13 different tumor types: gastrointestinal tract ($n = 33$), thoracic ($n = 24$), neurological ($n = 12$), breast ($n = 11$), genitourinary ($n = 8$), skin ($n = 8$), soft tissue ($n = 7$), gynecological ($n = 7$), head and neck ($n = 6$), bone ($n = 2$), and other types ($n = 3$). A wide range of tumor cellularity (25% to 100%) was tested in this sample cohort used. A minimum tumor cellularity of 20% was defined as a cutoff for the samples. The selected tumor specimens and cell lines had known mutation profiles, as analyzed by NGS in our laboratory using either the Ion Torrent PGM (50-gene Ion AmpliSeq Cancer Hotspot panel V2; Thermo Fisher Scientific, Carlsbad, CA) or Ion Proton (409 Comprehensive Cancer Panel; Thermo Fisher Scientific).

Nucleic Acid Extraction

FFPE cell lines and tumor samples were used for DNA extraction. For tumor samples, hematoxylin and eosin–stained tissue sections were reviewed for tumor content, and the tumor area was circled. Specimens with a minimum of 20% tumor cellularity in the marked area were selected for study. Five unstained tissue sections (5 μm thick) were deparaffinized, and tumor tissue was microdissected manually. DNA extraction and purification were performed using the Pico Pure DNA Extraction Kit (Arcturus, Mountain View, CA) and Agentcourt AMPureXP Kit (Agentcourt Biosciences, Beverly, MA), respectively. Extracted DNA and RNA were quantified using Qubit DNA HS Assay Kit and Qubit RNA HS Assay Kit, respectively (Thermo Fisher Scientific). The DNA and the RNA inputs required for the assay were 20 ng FFPE DNA (6 μL at a concentration of 3.3 ng/μL) and 10 ng FFPE RNA (5 μL at a concentration of 2 ng/μL), respectively. DNA extraction from 24 peripheral blood samples was performed using ReliaPrep Large Volume HT gDNA Isolation System (Promega, Madison, WI). RNA from tumors was extracted using FormaPure Kit (Agentcourt Biosciences), and RNA from cell lines was extracted using AllPrep FFPE DNA and RNA extraction kit (Qiagen, Valencia, CA).

Library Preparation for Sequencing

Library preparation for each sample was performed using the OCA (Thermo Fisher Scientific) following manufacturer's protocol. The panel includes two multiplexed primer pools that use 10 ng of DNA each (total, 20 ng) to amplify approximately 2530 genomic areas of interest in 143 genes. Sequencing library preparation was performed using the Ion Ampliseq Library Kit 2.0 (Thermo Fisher Scientific) according to the manufacturer's instructions. To facilitate multiplexed sequencing, sample barcoding was accomplished using the Ion Express Barcode Adapters 1-96 Kit (Thermo Fisher Scientific). The prepared library was quantified using the Ion TaqMan Quantitation Kit (Thermo Fisher Scientific). Pooled libraries of 24 multiplexed tumor samples