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Molecular Diagnostic Profiling of Lung Cancer Specimens with a Semiconductor-Based Massive Parallel Sequencing Approach

Feasibility, Costs, and Performance Compared with Conventional Sequencing

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In the context of personalized oncology, screening for somatic tumor mutations is crucial for prediction of an individual patient's response to therapy. Massive parallel sequencing (MPS) has been suggested for routine diagnostics, but this technology has not been sufficiently evaluated with respect to feasibility, reliability, and cost effectiveness with routine diagnostic formalin-fixed, paraffin-embedded material. We performed ultradeep targeted semiconductor-based MPS (190 amplicons covering hotspot mutations in 46 genes) in a variety of formalin-fixed, paraffin-embedded diagnostic samples of lung adenocarcinoma tissue with known *EGFR* mutations ($n = 28$). The samples reflected the typical spectrum of tissue material for diagnostics, including small biopsies and samples with low tumor-cell content. Using MPS, we successfully sequenced all samples, with a mean read depth of 2947 reads per amplicon. High-quality sequence reads were obtained from samples containing $\geq$10% tumor material. In all but one sample, variant calling identified the same *EGFR* mutations as were detected by conventional Sanger sequencing. Moreover, we identified 43 additional mutations in 17 genes and detected amplifications in the *EGFR* and *ERBB2* genes. MPS performance was reliable and independent of the type of material, as well as of the fixation and extraction methods, but was influenced by tumor-cell content and the degree of DNA degradation. Using sample multiplexing, focused MPS approached diagnostically acceptable cost rates. *(J Mol Diagn 2013, 15: 765–775; http://dx.doi.org/10.1016/j.jmoldx.2013.06.002)*

As the era of personalization in cancer medicine approaches, clinically relevant genetic alterations are increasingly used to stratify patients for specific targeted therapeutics.[1] This development began with the discovery of *ERBB2* (alias *HER2*, *NEU*) gene amplifications in breast cancer, which predict clinical response to trastuzumab.[2] Additional examples of novel genetics-driven cancer treatment include the mandatory detection of *BRAF* V600E mutations before use of vemurafenib for metastatic malignant melanoma[3] and therapeutic targeting of *EGFR* in both lung cancer[4] and colorectal cancer.[5] It is estimated that many more therapeutic approaches will become available in the near term to address the specific genetic patterns of a given tumor on a therapeutic level. This holds particularly true for lung cancer, for which additional therapeutic targets have been identified, including *MET*, *FGFR1*, *PIK3CA*, and *DDR2*.[6,7] With the recent advent of massive parallel sequencing (MPS), it is now

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possible to rapidly uncover genomic alterations in tumors in unprecedented detail and with relatively low cost. Data derived from recent consortium-based whole-genome and whole-exome sequencing efforts for many solid tumors have broadened our view of cancer as a complex genetic disease harboring an astounding heterogeneity.[8]

All of these studies relied on ample amounts of fresh-frozen tissue. In routine diagnostics, however, the available material frequently consists of only small biopsies, or even single clusters of neoplastic cells. In addition, tissue available for routine molecular diagnostics is likely to consist almost exclusively of formalin-fixed, paraffin-embedded (FFPE) material.[9] Whole-exome or even whole-genome sequencing of FFPE material is subject to various technical constraints,[10] and, although prices have dropped dramatically, it is not yet cost effective in a routine clinical setting. Nonetheless, MPS technologies have their place in sequencing of gene panels that focus on specific hot-spot mutations in a given number of genes that are either of differential diagnostic relevance or are known to be prognosticators or response predictors for available therapeutic approaches.

Such panels suffice in a routine diagnostic setting, because large-scale, whole-genome sequencing has revealed that, in a given tumor entity, only a moderate number of genes harbor mutations in >1% of the respective tumors, and of these only a few contain valuable prognostic, predictive, or differential diagnostic information. A broadened targeted approach may therefore be equally useful during routine practice as in cohort-based translational research studies. To date, only a few studies have applied MPS to routine diagnostic FFPE material, and the applications have been primarily exploratory.[11–15] We know of no published studies that comprehensively investigated the application of MPS methods to FFPE lung cancer tissue in a routine diagnostic setting. Such a setting is of specific clinical interest, because lung cancer is the solid tumor for which multigene testing is most likely to be applied in the near future. Additionally, only limited data are available on the validity and robustness of Ion Torrent (a recently introduced semiconductor-based MPS platform[16]; Life Technologies, Darmstadt, Germany; Carlsbad, CA) for FFPE cancer specimens.[17–19]

We investigated the validity of the Ion Torrent semiconductor-based MPS system for detecting Sanger-sequenced mutations in DNA derived from FFPE lung cancer specimens with respect to different DNA extraction methods, age of the probes, sample sizes (biopsies and resections), and tumor-cell content. We also made cost and hands-on time estimates, an important cornerstone for the incorporation of MPS platforms into daily routine diagnostics.

Materials and Methods

Study Design

We tested the reliability and validity of an MPS amplicon sequencing approach using DNA extracted from FFPE material. Although FFPE material is the basis for routine pathological diagnostics, specimens can vary greatly with respect to tissue size (biopsy versus resection), tumor-cell content, fixation time, block age, and methods of DNA extraction. To reflect these parameters, we selected a variety of specimens (five biopsy and seven resection specimens with a tumor-cell content of ≥50% but with differing block ages; four biopsies each with <10%, 10% to 25%, and 25% to 50% tumor-cell content), including samples that were processed and embedded outside our institution. In addition, we repeated the sequencing of two samples using half the volume of amplification reagent, and we tested two samples using different extraction methods (Table 1). To test the reliability of the sequencing runs with respect to detection accuracy, five samples were run in duplicate. For all samples, the presence of *EGFR* gene mutations had been confirmed by Sanger sequencing.

DNA extracted from FFPE material is degraded to various degrees, which limits its usefulness for sequencing approaches using classical capture methods. However, these DNA probes are routinely used for approaches such as classical Sanger sequencing, which depend on amplification of target regions by PCR. We therefore considered an amplicon-based approach to be best for generating MPS libraries. We used an Ion AmpliSeq cancer panel (Life Technologies), that contained primers for the amplification of 190 amplicons located in known hotspot cancer regions across the human genome. The median amplicon size generated by this approach was between 80 and 100 bp, which is small enough for use with significantly degraded DNA from FFPE material.

Tumor Material and DNA Extraction

For this study, FFPE non-small cell lung cancer (NSCLC) specimens were used ($n = 28$). All specimens represented routine diagnostic material that had been found, in the process of diagnostic workup, to harbor *EGFR* mutations. The specimens were selected from the archives according to the following criteria: coming from different hospitals with differing pathology labs, representing different types of material (resection specimen, biopsies), and containing different amounts of tumor cells (Table 1). Characterization of the tissue is further detailed under *Results*. All cases were diagnosed or rediagnosed by two experienced pulmonary pathologists (W.W. and A.W.) according to the criteria of the World Health Organization classification of lung cancer[20] and the current International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society consensus classification.[21]

Tumor areas were marked on an H&E–stained slide, and corresponding tissue areas were microdissected from subsequent unstained slides. Extraction of genomic DNA was performed by proteinase K digestion and fully automated purification using either a QIAsymphony SP system (Qiagen, Hilden, Germany; Valencia, CA) or a Maxwell 16