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Study of Preanalytic and Analytic Variables for Clinical Next-Generation Sequencing of Circulating Cell-Free Nucleic Acid

Q41 Meenakshi Mehrotra,* Rajesh R. Singh,* Wei Chen,* Richard S.P. Huang,†‡ Alaa A. Almohammedsalim,* Bedia A. Barkoh,* Crystal M. Simien,* Marcos Hernandez,* Carmen Behrens,§ Keyur P. Patel,* Mark J. Routbort,* Russell R. Broaddus,† L. Jeffrey Medeiros,* Ignacio I. Wistuba,¶ Scott Kopetz,‖ and Rajyalakshmi Luthra*

Q1 *From the Departments of Hematopathology,* Pathology,† Thoracic/Head and Neck Medical Oncology,§ Translational Molecular Pathology,¶ and Gastrointestinal Medical Oncology,‖ the University of Texas MD Anderson Cancer Center, Houston; and the Baylor College of Medicine,‡ Houston, Texas*

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Address correspondence to Rajyalakshmi Luthra, Ph.D., Department of Hematopathology, University of Texas MD Anderson Cancer Center, Unit 1062, 6565 MD Anderson Blvd, Houston, TX 77030. E-mail: rluthra@mdanderson.org.

Detection of mutations in plasma circulating cell-free DNA (cfDNA) by next-generation sequencing (NGS) has opened up new possibilities for monitoring treatment response and disease progression in patients with solid tumors. However, implementation of cfDNA genotyping in diagnostic laboratories requires systematic assessment of preanalytical parameters and analytical performance of NGS platforms. We assessed the effects of peripheral blood collection tube and plasma separation time on cfDNA yield and integrity and performance of the Ion PGM, Proton, and MiSeq NGS platforms. cfDNA from 31 patients with diverse advanced cancers and known tumor mutation status was deep sequenced using targeted hotspot panels. Forty-five of 52 expected mutations and two additional mutations (*KRAS* p.Q61H and *EZH2* p.Y646F) were detected in plasma through a custom bioinformatics pipeline. We observed comparable cfDNA concentration/integrity between collection tubes within 16 hours of plasma separation and equal analytical performance among NGS platforms, with 1% detection sensitivity for cfDNA genotyping. *(J Mol Diagn 2017, ■: 1–11; http://dx.doi.org/10.1016/j.jmoldx.2017.03.003)*

Q4 Determining the presence of molecular abnormalities in solid tumors is useful for diagnosis, selection of appropriate therapy, and monitoring tumor burden. Currently, diagnostic biopsy tissue specimens serve as the major source for tumor genotyping. However, this approach has substantial limitations because of intratumoral heterogeneity, cost, time, and risk associated with testing multiple biopsy specimens.[1] Furthermore, the impaired medical condition of many patients with advanced cancer, the inaccessible location of some tumors, and logistical considerations limit the feasibility of obtaining a biopsy in many circumstances.

The so-called liquid biopsy, especially plasma, has emerged recently as an alternative to surgical biopsy that allows real-time assessment of molecular alterations in patients with solid tumors.[2] More important, liquid biopsies such as plasma are minimally invasive, being easily obtained through a simple blood draw, which makes their use relatively inexpensive and readily scalable. Moreover, liquid biopsy can provide temporal measurements of tumor burden and can identify specific mutations that arise during therapy, provide early evidence of recurrence, and highlight mechanisms that underlie resistance to therapy.[1,3]

Potential sources of tumor genetic information in the circulation include cell-free circulating tumor DNA (cfDNA), circulating tumor RNA, circulating tumor cells, and exosomes. Among these, cfDNA offers an attractive option as a diagnostic, predictive, and prognostic biomarker for assessing tumor genetic information because of its stability and easy availability.[4,5] Plasma cfDNA levels have been shown to correlate with tumor size, degree of tumor invasion, disease stage, survival, and disease progression under therapy.[6]

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cfDNA is fragmented to an average length of 140 to 170 bp and is present in limited quantities per milliliter of peripheral blood, of which only a fraction may be tumor-derived DNA with diagnostically relevant mutations.[7,8] Various methods have been developed to detect the presence of low-level tumor-associated mutations in cfDNA of cancer patients.[1] However, clinical implementation of these cfDNA-based mutation tests has been impeded by a lack of robust preanalytical, analytical, and clinical validation studies. A comparison of frequently used next-generation sequencing (NGS)–based platforms in molecular diagnostic laboratories with respect to analytical sensitivity and specificity for cfDNA mutation analysis is lacking. In addition, variability in blood collection and handling can have substantial effects on quantitative measurement of cfDNA. For instance, cell lysis after venipuncture when using standard PB collection tubes necessitates separation of plasma within a short period after collection to reduce contamination of cfDNA with cellular DNA and increase the chances of detecting low-level tumor-associated mutations.[9,10]

For these reasons, PB collection tubes with cell-stabilization agents that prevent cell lysis for several days and alleviate the need for immediate plasma preparation after PB collection are recommended. However, changing from routinely used EDTA-based PB collection tubes to cell-stabilizing tubes for cfDNA molecular analysis requires a process change in phlebotomy units and adds costs to the testing. A systematic analysis of the impact of preanalytical variables, such as PB collection tubes and time interval from collection of PB to plasma separation on downstream mutation testing, is required before cfDNA genotyping assays can be implemented in molecular diagnostic laboratories for patient care.

In this study, we assessed the effect of different collection tubes on cfDNA yield and integrity and on downstream mutation analysis by gene panels on three NGS-based platforms (Ion PGM, Ion Proton, and MiSeq). We also compared the sensitivity and specificity of these three NGS platforms, which are widely used in molecular diagnostic laboratories, for detection of somatic mutations in cfDNA using a custom bioinformatics workflow in a clinical laboratory environment. To our knowledge, this is the first study to assess in parallel the impact of routinely used EDTA blood collection tubes versus cell-stabilizing collection tubes on cfDNA mutation analysis using NGS platforms for implementation in a Clinical Laboratory Improvement Amendments–certified laboratory environment.

Materials and Methods

Study Cohort

The study group included 31 patients with diverse advanced cancers and known tumor mutation status (Supplemental Table S1). The tumor mutations were identified using DNA derived from fixed, paraffin-embedded tumor tissue sections by using the semiconductor-based Ion PGM NGS platform with Ampliseq Cancer Hot Spot Panel v2 performed in our Clinical Laboratory Improvement Amendments–certified molecular diagnostic laboratory. The tumors included 28 carcinomas and 3 brain tumors. The carcinomas comprised eight endometrial adenocarcinomas (26%), seven colon adenocarcinomas (23%), three breast adenocarcinomas (10%), three prostate adenocarcinomas (10%), one ovarian carcinoma (3%), one lung adenocarcinoma (3%), one liver adenocarcinoma (3%), one esophagus signet ring adenocarcinoma (3%), one appendiceal mucinous adenocarcinoma (3%), one squamous cell carcinoma of the tongue (3%), and one parathyroid carcinoma (3%). The brain tumors were astrocytoma or ganglioneuroblastoma (10%). Tumor samples were obtained by resection (55%), biopsy (32%), or fine-needle aspiration (13%) from a variety of anatomical sites: lymph node (26%), liver (23%), abdominal tissue (10%), rectum (10%), brain (6%), uterus (6%), ovary (3%), prostate (3%), head and neck (3%), colon (3%), femur (3%), and esophagus (3%). There were 14 primary and 17 metastatic tumors. The patients had undergone various modalities of treatment before tumor specimens were obtained, including chemotherapy ($n = 5$), chemotherapy and radiation ($n = 2$), surgery and chemotherapy ($n = 17$), or surgery, chemotherapy, and radiation ($n = 7$). This study protocol was approved by the Institutional Review Board of MD Anderson Cancer Center and is consistent with international ethical standards on human subjects research. Informed consent was obtained from each study participant.

Sample Collection

Peripheral blood was drawn from each patient at the same time into two different blood collection tubes: a regular K3-EDTA tube (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ) and a Cell-Free DNA BCT tube (Streck, Inc., Omaha, NE), which contains cell-stabilizing agents that prevent cell lysis.

cfDNA Extraction and Quantification

Blood samples were mixed by inverting tubes 10 times and subjected to centrifugation at 2000 × *g* for 10 minutes at room temperature. Plasma was separated from blood at different time points (2 to ≥24 hours). The plasma layer was carefully removed without disturbing the buffy coat, transferred to a new vial, and subjected to centrifugation at 2000 × *g* for 10 minutes at room temperature to remove any residual cells. cfDNA was extracted from a 3-mL plasma sample using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA) by following the manufacturer's instructions. Elution was performed in 50 μL, and isolated cfDNA was kept at −20°C. DNA was quantified by using the Qubit dsDNA HS Assay (Life Technologies, Illkirch, France).

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