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ORIGINAL ARTICLE

Challenges in next generation sequencing analysis of somatic mutations in transplant patients

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Abstract

Analysis of somatic mutations in solid tumors and hematologic malignancies using targeted next generation sequencing (NGS)-based assays has become part of routine oncology practice as well as clinical trials. The use of paired tumor-normal DNA samples increases confidence of somatic calls. NGS assays that utilize unique patient identifiers (SNP IDs) allow further comparison of samples within a run or paired tumor/normal samples. The sources of germline DNA include peripheral blood (PB) and formalin-fixed paraffin-embedded tissue (FFPE). However, the source of normal can be problematic, especially in transplant setting. Herein, we report two cases of NGS-based molecular testing in a patient with mycosis fungoides treated with stem cell transplant [SCT] (Pt1) and a patient with lung adenocarcinoma who previously had acute leukemia cured by SCT. These cases highlight the importance of selecting an appropriate normal sample for excluding germline polymorphisms during somatic mutation testing. Initial analyses that included concurrent PB sample failed to filter known germline polymorphisms. Repeat analyses using pre-transplant PB/bone marrow allowed for the successful subtraction of germline variants. Somatic mutations in *PTEN* and *ERBB4* (Pt1) and *CDKN2A, KRAS, KDR,* and *TP53* (Pt2) were reported with confidence. Selection of an appropriate source of germline DNA for NGS-based somatic mutation testing for patients with SCT transplant can be challenging. Particular attention to the clinical history is crucial for accurate interpretation and reporting.

Keywords Solid tumor testing, NGS, Somatic mutations, Germline subtraction, Transplant.
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Introduction

Analysis of somatic mutations in solid tumors and hematologic malignancies using next generation sequencing (NGS)-based assays has now become part of routine oncology practice as well as clinical trials [1–6]. NGS is an important tool in personalized cancer therapy aiming at delivering maximum therapeutic benefits while minimizing therapy-associated risks. The clinically adopted genomic analyses include hot spot mutation panels and, less frequently, whole exome sequencing [7–10]. With a constantly growing list of actionable mutations, accurate classification of tumor-specific (i.e. somatic) and germline variants becomes increasingly important in determining eligibility for targeted therapies and need for referral for genetic counseling with potential implication not only for the patient but also for the patient's family [11,12].

Formalin-fixed paraffin-embedded (FFPE) tissue samples available in the files of anatomic pathology departments are an accepted source of tumor DNA. Additional pre-genomic testing histological review geared to selection of the optimal

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tumor block containing sufficient tumor cellularity to match the sensitivity of the assay provides an estimated percentage of tumor cells in the sample. This parameter is incorporated in the interpretation of the molecular findings. One of the challenges in interpreting and reporting molecular findings is related to establishing somatic vs. germline status of the discovered alterations especially when testing includes only tumor specimen. Several approaches used concurrently that allow for interpretation of the data derived from analysis of the tumor-only DNA include bioinformatic filtering using publicly available databases (dbSNP, Catalogue of Somatic Mutations in Cancer [COSMIC], etc.) and the evaluation of the fraction of mutant allele in conjunction with estimated tumor cellularity. Use of the germline databases may occasionally lead to inadvertent filtering of somatic variants. The latter approach relies on the expected germline variant allele fraction being close to 50% and 100% for the heterozygous and homozygous variants, respectively. This assumption can be useful in predicting germline vs. somatic variant origin in cases with estimated tumor fraction of less than 50% leading to detection of sub-heterozygous mutant allele fraction for the somatic mutations; it becomes less reliable in samples with greater than 50% tumor cellularity [13]. Parallel sequencing of tumor and normal DNA significantly increases confidence in assigning somatic origin to the discovered alterations [13]. Peripheral blood (PB) is generally considered a preferred source of germline DNA. Alternatively, DNA from saliva can be used. In the instances when neither type of specimen is readily available, which is not infrequent in a tertiary cancer care centers serving a geographically diverse patient population, FFPE normal tissue is often used [14]. In such cases, careful review of the corresponding H&E is required to ensure lack of the tumor in the tissue block used for germline filtering.

Stem cell transplantation (SCT) is becoming increasingly used in a variety of constitutional conditions and hematologic malignancies. This intervention alters genomic makeup of an individual peripheral blood and leads to presence of two unique “germline” complements: one the patient’s original and the other derived from the donor. Selection of an appropriate source of germline DNA for patients with stem cell transplant can be problematic. To illustrate somatic mutation analysis challenges in transplant setting, herein, we report testing in two SCT patients. One patient (Pt 1) had therapy refractory mycosis fungoides (MF); the other patient (Pt 2) was diagnosed with lung adenocarcinoma and had a history of mixed phenotype acute leukemia cured by SCT.

Materials and methods

The study was approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center. Mutational analysis of the tumor(s) tissue was requested by a clinical team as a part of patient care. In our practice composed of patients with advanced solid tumors, multiplex NGS analysis is performed in search for potentially actionable somatic alterations to determine eligibility for standard therapy or enrollment in targeted therapy clinical trial. The informed consent includes text regarding potential discovery of significant germline findings. However, as this test is not intended as a substitute for formal comprehensive germline testing, only somatic mutations are reported. In cases in which potentially significant germline findings are discovered, the genetic counseling service is contacted to inform the patient of a potential need for further work-up. It is then up to the patient to decide if he/she would like to pursue formal germline testing to confirm the presence of the deleterious germline mutation.

Tissue selection and DNA extraction

Hematoxylin and eosin (H&E) stained tissue sections of FFPE tumor in the skin and lung biopsy for patients 1 and 2, respectively, were reviewed and the tumor area was circled by a pathologist to ensure that a sample with a minimum of 20% tumor cells was used for analysis. The consecutive unstained tissue sections of 5 μM thickness were deparaffinized and manually dissected using the H&E stained slide as a guide. DNA extraction and purification were performed using the Pico Pure DNA Extraction Kit (Thermo Fisher Scientific, Waltham, MA). Extracted DNA was analyzed using Qubit DNA HS Assay Kit (Thermo Fisher Scientific). DNA extraction from peripheral blood sample (a default source of germline DNA) was performed using ReliaPrep Large Volume HT gDNA Isolation System (Promega, Madison, WI).

Next generation sequencing

Paired tumor DNA derived from FFPE tumor tissue and control germline DNA from PB sample or FFPE normal tissue were used for mutational analysis in each patient. Mutational analysis was performed using the 134-gene Oncomine cancer panel (Thermo Fisher Scientific) following the manufacturer’s protocol as previously described [9]. The DNA input required for the assay was 20 ng FFPE DNA. Thus, 20 ng of DNA was used to amplify approximately 2530 genomic areas of interest in 134 genes. Sequencing library preparation was performed using the Ion Ampliseq Library Kit 2.0 (Thermo Fisher Scientific). 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 (normal–tumor paired samples) with sensitivity intra-run control at a concentration of 10 pmol were used for clonal amplification on beads by emulsion PCR using Ion PI Hi-Q OT2 200 Kit (Thermo Fisher Scientific). Sequencing on the Ion Proton sequencer was performed with Ion PI Hi-Q Sequencing 200 Kit and Ion Chip PI Kit version 3 (Thermo Fisher Scientific).

Sequencing data analysis

Sequencing data analysis was performed according to our standard laboratory procedures as describe previously [9]. In brief, Torrent Suite software version 4.4.3, Variant Caller version 4.4.2.2 and Coverage Analysis version 4.4.3.3 plug-ins (Thermo Fisher Scientific) were used for raw data, variant calling and sequencing coverage analysis, respectively. A minimum sequencing depth of 250 was considered as adequate sequencing depth (for the amplicon). The cutoff for variant allele fraction [variant coverage (numerator) divided by sequencing depth (denominator)] of 5% was established based

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