Complexities of Next-Generation Sequencing in Solid Tumors: Case Studies

Alexandra O. Sokolova, MD^{1,2,3}; Brian H. Shirts, MD, PhD⁴; Eric Q. Konnick, MD, MS⁴; Ginger J. Tsai, MS⁴; Bernardo H.L. Goulart, MD^{1,2}; Bruce Montgomery, MD^{1,2,3}; Colin C. Pritchard, MD, PhD⁴; Evan Y. Yu, MD^{1,2}; and Heather H. Cheng, MD, PhD^{1,2}

ABSTRACT

With the promise and potential of clinical next-generation sequencing for tumor and germline testing to impact treatment and outcomes of patients with cancer, there are also risks of oversimplification, misinterpretation, and missed opportunities. These issues risk limiting clinical benefit and, at worst, perpetuating false conclusions that could lead to inappropriate treatment selection, avoidable toxicity, and harm to patients. This report presents 5 case studies illustrating challenges and opportunities in clinical next-generation sequencing interpretation and clinical application in solid tumor oncologic care. First is a case that dissects the origin of an *ATM* mutation as originating from a hematopoietic clone rather than the tumor. Second is a case illustrating the potential for tumor sequencing to suggest germline variants associated with a hereditary cancer syndrome. Third are 2 cases showing the potential for variant reclassification of a germline variant of uncertain significance when considered alongside family history and tumor sequencing results. Finally, we describe a case illustrating challenges with using microsatellite instability for predicting tumor response to immune checkpoint inhibitors. The common theme of the case studies is the importance of examining clinical context alongside expert review and interpretation, which together highlight an expanding role for contextual examination and multidisciplinary expert review through molecular tumor boards.

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Background

Over the past decade, advances in next-generation sequencing (NGS) have decreased cost and enabled more widespread use in solid tumor oncology. This has been accompanied by increasing therapeutic options based on tumor genomic profiling, such as *ROS1* mutations in lung cancer¹ and *BRAF* mutations in melanoma.² In addition, the clinical applications of germline sequencing in solid tumors have rapidly expanded.^{3–6} Thus, NGS is a powerful tool that can potentially provide an indication for targeted therapies and inform prognosis and family cancer risks. However, the interpretation of comprehensive NGS findings can be challenging and complex and can raise additional questions, even as it provides answers. This report presents several case studies illustrating important considerations in NGS testing in the solid tumor oncology setting.

Interference of Clonal Hematopoiesis in Somatic Sequencing

The first case involved an 80-year-old man with metastatic castration-resistant prostate cancer (mCRPC) whose disease was previously treated with abiraterone acetate and progressed on enzalutamide. He underwent biopsy of a bone metastasis in November 2018 with the hope of identifying potentially actionable mutations for additional treatment options. FoundationOne CDx (Foundation Medicine) testing of biopsy material revealed a well-studied *ATM* c.9022C>T (p.R3008C) pathogenic mutation⁷ at 10% allelic fraction, but the sample was insufficient to assess microsatellite instability (MSI) or tumor mutational burden. The patient was seen in the clinic to discuss germline genetic testing and, as a result of the reported *ATM* mutation, consider targeted therapy clinical trial options, including several involving PARP inhibitors. Given the patient's interest in immune checkpoint inhibitors, a targeted gene NGS panel (UW-OncoPlex) validated for circulating tumor DNA (ctDNA) was sent to assess for MSI and tumor mutational burden in September 2019. The sample had a positive test result for

¹Department of Medicine, University of Washington; ²Clinical Research Division, Fred Hutchinson Cancer Research Center; ³VA Puget Sound Health Care System; and ⁴Department of Laboratory Medicine, University of Washington, Seattle, Washington.

2 presumed somatic mutations in *ATM*, c.9022C>T (p.R3008C) at 12% variant fraction and c.9021A>T (p.E3007D) at 5% variant fraction, and these were noted in *trans* (on different alleles). A peripheral blood DNA study shared the same *ATM* c.9022C>T (p.R3008C) mutation reported in the prostate cancer metastatic bone biopsy, but the *ATM* c.9021A>T (p.E3007D) mutation had not previously been reported. It was discovered that for ctDNA testing, whole blood had inadvertently been drawn instead of plasma, the result of which was that lymphocytes constituted most of the DNA and would be expected to overwhelm the much smaller proportion of tumor-specific ctDNA. To clarify findings, plasma ctDNA (in which circulating cells are removed by centrifugation) was drawn. The same 2 mutations in *ATM* were identified: c.9022C>T (p.R3008C) at 16% variant fraction and c.9021A>T (p.E3007D) at 5% variant fraction. The plasma cell-free DNA was compared with the reported results from the bone metastasis biopsy and whole blood. Although the first *ATM* c.9022C>T (p.R3008C) mutation was identified in all 3 samples, the variant allelic fraction of the tumor-specific somatic variant should be higher in the plasma sample than in the whole blood sample and highest in the metastatic bone biopsy (Figure 1). However, this was not observed. Careful review of the variant allelic fractions from the 3 studies together showed that they were consistent with a clonal hematopoietic (CH) process being the likely source of the 2 *ATM* clones and, importantly, unrelated to the prostate cancer.

Both ctDNA and metastatic biopsies can potentially be contaminated by CH and may be detected as minor populations in clinical NGS assays. Increasing evidence suggests that CH is a source of interference and should be routinely considered during interpretation of solid tumor NGS studies.⁸⁻¹⁴ A prospective study in which 124 patients with metastatic cancer underwent paired NGS of peripheral blood and tumor DNA with high-intensity sequencing (508 genes, 2 Mb, >60,000× raw depth) reported that 53% (918/1,727) of mutations in ctDNA samples were attributable to CH.¹⁴ A large retrospective analysis of samples from patients with solid tumors reported that in the absence of matched blood control subjects, 5% (912/17,469) of patients would have had at least one CH-associated mutation identified from ctDNA erroneously attributed to the solid tumor.⁸ The most frequently altered genes in CH were *DNMT3A*, *TET2*, *PPM1D*, *ASXL1*, *TP53*, *SF3B1*, *SRF2*, *CHEK2*, *ATM*, and *KMT2D*.⁸ In another study, *ATM* mutations were deemed of likely CH origin in 20.9% (162 of 775) of individuals undergoing hereditary pan-cancer panel testing.⁹ Distinguishing between CH and cancer-associated mutations is of great clinical importance in both metastatic biopsies and ctDNA assays.

Different tumor specimen types can be used for NGS analysis, each with advantages and disadvantages (Table 1). This case illustrates how assessment of multiple samples, combined with expert interpretation and molecular tumor board review, was critical and allowed the patient to avoid a treatment targeting *ATM* mutations, which may have been ineffective due to interpretive analytic error rather than a bona fide tumor-specific driver mutation.

Somatic Versus Germline

The second case involved a 76-year-old man with high-risk localized prostate cancer (cT2b; Gleason score 4 + 4 = 8; prostate-specific antigen [PSA] level, 9.1 ng/mL). This patient's family history was notable for an older sister diagnosed with breast cancer at age 73 years and his mother diagnosed with mesothelioma at age 80 years. He had no other family history of cancer, including among another sister and 2 daughters. The patient was part of a research study in which tumor DNA was evaluated using a comprehensive targeted gene NGS panel (UW-OncoPlex). The panel results revealed biallelic *BRCA2* mutation: *BRCA2* c.9196C>T (p.Q3066×) at 41% variant allelic fraction and *BRCA2* c.7914del (p.P2639Lfs*9) at 29% variant allelic fraction. The discovery of *BRCA2* alterations, particularly at higher variant allelic fraction, prompted genetic counseling referral. Dedicated germline genetic testing confirmed that the *BRCA2* c.9196C>T was a heterozygous germline variant and led to cascade genetic testing of his family. His older sister with breast cancer and one of his 2 daughters were found to carry the same germline *BRCA2* c.9196C>T mutation. These findings changed management in his sister's breast cancer treatment, his daughter's cancer surveillance, and finally the patient's own surveillance for other cancers. He completed definitive radiation therapy with androgen deprivation therapy (ADT) and currently has no signs of recurrence.

Both germline and somatic genetic testing may inform treatment decisions and prognosis for patients with cancer. Identifying potential germline mutations from tumor NGS can be clinically important. Reporting of suspected germline mutations varies across commercially available NGS platforms, with some laboratories reporting findings that may potentially be germline, other laboratories reporting both tumor and germline findings in aggregate and not calling attention to results that could be germline, and still other laboratories with bioinformatics set up to filter out germline data, thus removing the ability to identify germline variants. Clues that suggest potential germline origin of variants are relatively high variant allelic fraction (eg, ~40%), previous studies of variants in germline capacity (eg, ClinVar), and strong family history of cancer.

Figure 1. Conceptual model of VAF percentage in whole blood, plasma, and bone biopsy (with tumor purity >80%). Germline variants would be expected to have the same VAF in whole blood, plasma, and bone biopsy. Somatic tumor variants are expected to have significantly lower VAF in whole blood than in plasma because all blood cells are contributing to the denominator in whole blood samples and cells are removed in the plasma sample. Hematopoietic variants are expected to have the same VAF in whole blood and plasma.

Abbreviations: ctDNA, circulating tumor DNA; VAF, variant allelic fraction.

It should be noted that because of these differences in reporting across assays, the absence of germline variants/mutations on somatic testing is insufficient to make germline conclusions. Genetic counseling and dedicated germline testing are recommended if tumor NGS identifies a pathogenic variant in a gene associated with a cancer predisposition gene, especially if observed

at high variant allelic fraction or there is other suspicion based on personal or family history. A special note of caution regarding *TP53*, which is a gene very commonly mutated in both CH and tumors and is rarely representative of the germline Li-Fraumeni syndrome: routine germline testing is not necessary for most patients with somatic *TP53* mutations and should be reserved only for patients

Table 1. Comparison of DNA Sources for Somatic Next-Generation Sequencing

Sample	Limitations	Advantages
Plasma ctDNA	Sensitivity depends on context (correlates with tumor burden) Specificity is uncertain (may reflect noncancer processes; eg, CH)	Minimally invasive May reflect composite of multiple progressing clones
Primary tumor biopsy	May not reflect changes arising later in oncogenesis (truncal/driver events may be detectable)	If available, no additional invasive procedure is required
Metastasis biopsy	Invasive procedure required May not reflect tumor heterogeneity	May reflect dominant or progressing clonal population

Next-generation sequencing for CTCs is not clinically used. CTC enumeration is FDA-approved as a prognostic tool in metastatic breast, colorectal, and prostate cancer. CTCs are also being investigated for single-cell analysis, RNA sequencing, and prediction of response/recurrence.

Abbreviations: CH, clonal hematopoiesis; CTC, circulating tumor cell; ctDNA, circulating tumor DNA.

with suspicious personal/family history and with genetic counseling involvement.

In this patient's case, expertise in tumor-germline NGS interpretation allowed recognition that the results were concerning for a possible germline *BRCA2* variant. This led to a referral to genetic counseling, confirmatory germline testing, and cascade family testing that identified the mutation in his sister and daughter and ultimately provided valuable information for both the patient and his family. This case also illustrates that men with prostate cancer can be the first in the family to be identified as carrying germline mutations such as *BRCA2* and that cascade testing of relatives can be clinically important.

Reclassification of Variants of Unknown Significance

Reclassification to Likely Benign

The third case involved a 48-year-old woman diagnosed with breast cancer. She underwent germline testing and was found to have c.2658G>T (p.Q866H) variant of unknown significance (VUS) in *APC*. On genetic testing, her 75-year-old mother was found to have the same VUS in *APC*. After learning about the *APC* VUS, the mother had an age-appropriate colonoscopy—her first colonoscopy—which did not identify colon polyps, arguing that the *APC* variant was unlikely to cause clinically actionable risk of polyposis. *APC* phenotype is highly penetrant, so a 75-year-old carrier without a clinical phenotype provided evidence that *APC* c.2658G>T (p.Q866H) should be reclassified as likely benign. It is important to note that discordant phenotypes alone are typically insufficient to downgrade VUS, but they may provide stronger evidence in cases of highly penetrant genes.

With the increasing use of multigene panel germline testing, we anticipate that more variants will be reported as VUS, which are challenging for patients to understand and for providers to resist overinterpreting within a clinical context. The latest information on variant relationships to phenotypes with supporting evidence can be found in freely accessible online databases: COSMIC (Catalogue of Somatic Mutations in Cancer) for somatic variants (cancer.sanger.ac.uk/cosmic) and ClinVar for germline variants (www.ncbi.nlm.nih.gov/clinvar/). ClinVar provides current information on variant pathogenicity classification.

VUS reclassification occurs via collaborative and labor-intensive work and most commonly results in VUS reclassification to likely benign/benign.^{15,16} The Find My Variant Study reported that 38 (61%) of 63 variants were reclassified, with 32 (84%) reclassified as benign/likely benign and 6 (16%) as pathogenic/likely pathogenic.¹⁷

Through genetics professionals, patients with VUS can be encouraged to seek studies to help reclassify VUS, aid clinical management, and improve understanding of genotype–phenotype correlations. Studies can be found at ClinicalTrials.gov, such as the PROMPT study (ClinicalTrials.gov identifier: NCT02665195). Participating in clinical trials to reclassify VUS may be particularly important for patients with a strong family history of cancer, such as in the next case.

Reclassification to Likely Pathogenic

The fourth case involves a 50-year-old African American man diagnosed with T3aN1M0, Gleason 4 + 4 prostate cancer. His sister had been diagnosed with breast cancer at age 40 years; his father and paternal uncle with prostate cancer in their 50s and 60s, respectively; his paternal first cousin with breast cancer at age 55 years; and his paternal grandmother with ovarian cancer (age unknown). Based on his personal and family history of cancer, he underwent panel NGS germline testing with tumor control. He was found to carry a germline *BRCA2* VUS, specifically *BRCA2* c.8188G>C (p.A2730P).

The patient and his family were invited to participate in the Find My Variant research study to help reclassify the VUS.¹⁷ His sister with breast cancer had previously undergone germline testing and carried the same *BRCA2* VUS. Segregation studies and familial cosegregation analysis were performed through the research study, and his paternal first cousin was found to also carry the variant. This indicated that the patient's father, who was diagnosed with prostate cancer, and a paternal uncle not known to have cancer were obligate carriers of the *BRCA2* VUS. Integration of available data gave a 99% probability of *BRCA2* variant pathogenicity. *BRCA2* p.A2730P VUS was thus reclassified as likely pathogenic. Although the patient's family history was suggestive of a familial cancer syndrome and would prompt intensified cancer surveillance in family members, reclassification of *BRCA2* VUS expanded treatment options for the patient and his sister. This specific *BRCA2* variant is more commonly observed in African American individuals, so the variant reclassification will help other families. Although VUS reclassification to likely pathogenic is rare, when there is a strong family history of cancer, participation in research studies such as in this case can help define less commonly seen variants. Concerted efforts to improve diversity of representation of available genetic databases will also help VUS reclassification.

Molecular Markers for Response to Therapy

The fifth case involved an 83-year-old man with mCRPC who initially presented with a PSA level of 317 ng/mL, lymphadenopathy, and a bladder mass, which was biopsied to reveal Gleason score 5 + 4 prostate adenocarcinoma.

He initiated ADT and underwent tumor sequencing that showed MSI and hypermutation associated with biallelic *MSH2* mutation (exons 1–6 + *EPCAM* deletion). The result of germline testing for *MSH2/EPCAM* was negative. His prostate cancer developed resistance to ADT within 2 months and was refractory to both abiraterone acetate and enzalutamide. He received pembrolizumab as third-line treatment of mCRPC due to tumor MSI/hypermutation, but he did not achieve a clinical response to pembrolizumab.

The FDA approved pembrolizumab as the first tumor-agnostic therapy for patients with advanced solid tumor with deficient mismatch repair (dMMR) or MSI¹⁸ but did not specify a companion diagnostic or method used to determine dMMR/MSI. dMMR/MSI can be identified by immunohistochemistry, PCR, or NGS. In a retrospective series of 93 patients with colorectal cancer, 10% (n=9) were found to have a misdiagnosis of dMMR/MSI status by local assessment,¹⁹ highlighting the need for standardization of methods to determine dMMR/MSI. Possible discordance of results based on sample source and timing of the biopsy has been reported, suggesting that MSI/dMMR may develop later in the disease and be present in a metastatic tumor but not in the primary tumor.²⁰

As this case illustrates, not all MSI/dMMR tumors respond to immune checkpoint inhibitors. This is corroborated by other studies, which in aggregate suggest a response rate to checkpoint blockade of approximately 50% among patients with MSI/dMMR.^{20,21} Further work is needed and is ongoing regarding as-yet-undetermined factors that affect biologic consequences, primary resistance, and/or treatment response prediction. Models integrating multiple factors may perform better in predicting response to immune checkpoint inhibitors.

Conclusions

NGS is an emerging tool in clinical oncology. These case studies illustrate several key clinical and research considerations for interpretation of NGS results (Table 2):

- Mutations arising from contaminating hematopoietic clones may confound results from ctDNA and tumor/metastatic biopsies
- Tumor sequencing may raise suspicion for germline mutation and may call for confirmatory genetic counseling and testing
- Providers should be aware of potential follow-up opportunities for VUS reclassification, especially if there is a high pretest suspicion (eg, a strong family history of cancer)
- Shared expertise through molecular tumor boards may help in the accurate interpretation of complex NGS results after understanding the clinical context

The most extensive bioinformatics without clinical correlation and expert interpretation may lead to faulty conclusions and hamper clinical benefit. Recognition of the importance of these factors is reflected by emerging forums, such as the now-annual ASCO seminar, Genetics and Genomics for the Practicing Physician, first offered in 2018. Molecular tumor boards provide the opportunity for multidisciplinary discussion of patient medical history, family history, and NGS results to guide patient-specific treatment decisions, and should include faculty with expertise in genetics, laboratory medicine, pathology, and oncology. Commercial NGS platforms often provide access to their molecular tumor boards to discuss challenging cases. As NGS testing is increasingly deployed in clinical practice and data and informatics become denser and more complex, there is a need to create a network structure that will allow community

Table 2. Strategies and Approaches to Address Selected Issues With NGS

Issue	Strategies and Approaches
CH interference in ctDNA or tumor biopsy	Request VAF Interpret VAF in the context of available test results and clinical picture
Tumor NGS is not universally reliable for germline variant identification	Dedicated germline testing is required for questions about inherited risk
Tumor NGS may raise suspicion of germline variant	Counsel the patient on the possibility before somatic NGS If a germline variant is suspected, refer the patient to a genetic counselor for discussion and dedicated germline testing
VUS reclassification	VUS should not be used for clinical management (rather, family history of cancer should guide management) Most VUS reclassifications are to benign or to likely benign Suggest patient follow-up periodically for VUS reclassification Consider enrolling patient in studies for VUS reclassification, especially if a strong family history of cancer is present

Abbreviations: CH, clonal hematopoiesis; ctDNA, circulating tumor DNA; NGS, next-generation sequencing; VAF, variant allelic frequency; VUS, variant of unknown significance.

oncologists to have access to academic and tertiary care center expert molecular tumor boards and keep pace with rapidly evolving knowledge and understanding.

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Correspondence: Heather H. Cheng, MD, PhD, Department of Medicine, University of Washington, 825 Eastlake Avenue East, Seattle, WA 98109. Email: hhcheng@uw.edu

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